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Prevalence, underlying mechanisms and role of nutrition in the management of the HIV-associated lipodystrophy syndrome

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**Prevalence, underlying mechanisms and role of nutrition in
the management of the HIV-associated lipodystrophy
syndrome**

By Cathriona R Loonam

A thesis submitted to King's College London for the degree of
Doctor of Philosophy in the Faculty of Science

Diabetes and Nutritional Sciences Division
School of Medicine
King's College London
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For my parents, my siblings, nonna Rosaria
and Charlie

Publications and presentations

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“Differential effects of conjugated linoleic acid isomers on PPAR-gamma and adipocyte-specific genes in adipocytes pre-treated with antiretroviral drugs”. IUNS International Congress of Nutrition, Granada, Spain. 15th –20th September 2013. Poster presentation.

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Abstract

HIV-associated lipodystrophy syndrome (HALS) is characterised by abnormalities of body fat distribution and metabolism resulting from adipocyte dysfunction. This thesis examines the hypothesis that antiretroviral drugs (ARVs) alter PPAR- γ , which contributes to adipocyte dysfunction. We hypothesise that conjugated linoleic acid (CLA) isomers, as putative PPAR- γ ligands, will attenuate adipocyte dysfunction and HALS.

In vitro, ritonavir (RTV; 20 μ M) was the most potent ARV and significantly decreased triglyceride storage ($P < 0.001$), perilipin ($P = 0.003$) and Cidea expression ($P < 0.001$), PPAR- γ expression ($P = 0.014$) and nuclear binding ($P = 0.03$), and adiponectin secretion ($P < 0.001$) compared with control. Microarray analysis revealed adipocyte differentiation as a top pathway affected by RTV ($P = 0.007$).

The addition of both c9,t11 and t10,c12 (100 μ M) to RTV-treated cells *in vitro* significantly increased triglyceride accumulation compared with control ($P < 0.001$). c9,t11 increased PPAR- γ nuclear binding ($P = 0.038$), while t10,c12 decreased perilipin expression ($P = 0.004$) and adiponectin expression ($P = 0.038$) and protein secretion ($P = 0.003$). Although c9,t11 had positive effects on adipocyte function, t10,c12 enhanced the detrimental effects of RTV.

The prevalence of HALS was found to be 29% among a cohort of UK patients with HIV. Receiving a first line ARV regimen was associated with reduced odds of developing HALS (OR 0.28, $P = 0.003$).

A study was conducted to investigate the effect of CLA (3 g/d) *versus* placebo (high-oleic sunflower oil) on abdominal obesity in HIV-infected men. Five of 478 patients were eligible and completed the study. No difference in WC was detected between the groups after 12 weeks of supplementation. Information collected as part of this study will help inform future polyunsaturated fatty acid interventions among HIV patients. The findings of this thesis are discussed in the context of existing scientific literature in the area.

Author's contribution

The author conducted all studies outlined in this thesis.

Erick Nasser conducted the microarray analysis of RNA samples collected and prepared by the author. Tracy Dew analysed plasma adipocytokines in HIV-positive samples from the CLAART Study.

The author statistically analysed and interpreted data from all studies and composed the present thesis. All statistical analysis was conducted in discussion with Dr Anne Mullen. Mr Bolaji Coker, statistician, was consulted for advice on statistical analysis of the CREATE data. I am very grateful to Dr Mullen and Mr Coker for their advice and discussion throughout this work.

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Abbreviations

ADRB3	beta 3 adrenergic receptor
AIDS	acquired immune deficiency syndrome
AMP	adenosine monophosphate
ANOVA	analysis of variance
aP2	adipocyte lipid binding protein-2
ART	antiretroviral therapy
ARVs	antiretroviral drugs
ASP	acylation stimulating protein
BAT	brown adipose tissue
BIA	bioelectrical impedance analyser
BMD	bone mineral density
BMI	body mass index
BrCa	breast cancer
BSA	bovine serum albumin
Ca	Cancer
CC	case control
CCR5	C-C chemokine receptor type 5
CDC	Centre for Disease Control
cDNA	complementary DNA
CEBP	CCAAT / enhancer binding protein
CFD	adipsin
CHD	coronary heart disease
CI	confidence interval
CIDE	Cell death-inducing DFFA-like effector
CLA	conjugated linoleic acid
CLAART	CLA and ART Study
CPT1	carnitine palmitoyl transferase 1
CR	case report
CREATE	Cardiovascular Risk Evaluation and Antiretroviral Therapy Effects
CRP	C-reactive protein
CS	cross-sectional
CV	cardiovascular
CVD	cardiovascular disease
CCR5	C-C chemokine receptor type 5
C1QR	Complement component C1q receptor
C3	Complement C3
c9,t11	<i>cis</i> -9, <i>trans</i> -11 conjugated linoleic acid
DEPC	diethylpyrocarbonate
DHA	docosahexanoic acid
DXA	dual energy xray absorbtimetry
DHA	docosahexanoic acid
d4T	stavudine
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DPCT	double-blind placebo controlled trial
EACS	European AIDS Clinical Society
ECM	extracellular remodelling
EDTA	Ethylenediaminetetraacetic acid

EFSA	European Food Safety Authority
EFV	efavirenz
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EPA	eicosapentanoic acid
EU	European Union
ER	estrogen receptor
ERK	extracellular signal regulated protein kinase
FABP	fatty acid binding protein
FAME	fatty acid methyl ester
FASN	fatty acid synthase
FCS	foetal calf serum
FDA	Food and Drug Administration
FDR	false discovery rate
FFA	free fatty acids
FFQ	food frequency questionnaire
FG	fasting glucose
FMD	flow mediated dilatation
FMR	fat mass ratio
FTC	emtricitabine
GI	glycaemic index
GLC	gas liquid chromatography
GLUT-4	glucose transporter type 4
GPR	G-protein coupled receptor
HAART	highly active antiretroviral therapy
HALS	HIV-associated lipodystrophy syndrome
HbA1c	glycated haemoglobin
HBV	hepatitis B virus
HCV	hepatitis C virus
HDL-C	high density lipoprotein cholesterol
HIV	Human Immunodeficiency Virus
HMW	high molecular weight
HOSF	high oleic sunflower oil
HPRT	hypoxanthine-guanine phosphoribosyltransferase
HRP	horseradish peroxidase
HSL	hormone sensitive lipase
HTN	hypertension
hs-CRP	high sensitivity C-reactive protein
IBMX	3-isobutyl-1-methylxanthine
IDF	International Diabetes Federation
IDV	indinavir
IFN- γ	interferon-gamma
IGF	insulin-like growth factor
IL	interleukin
INSIG	insulin-induced gene 1
IR	insulin resistance
IRS	insulin receptor substrate
IQR	interquartile range
IVDU	intravenous drug user
LA	lipoatrophy
LBM	lean body mass
LD	lipodystrophy

LDL-C	low density lipoprotein cholesterol
LH	lipohypertrophy
LPL	lipoprotein lipase
LPS	lipopolysaccharide
LXR	liver-X-receptor
MAPK	mitogen activated protein kinase
MCP	monocyte chemoattractant protein
MDA	malondialdehyde
MET	metabolic equivalents
MetS	metabolic syndrome
MI	myocardial infarction
MMP	matrix metalloproteinase
n-3 PUFA	omega 3 polyunsaturated fatty acid
NCC	nested case control
NCEP	National Cholesterol Education Programme
NF- κ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NRTI	nucleoside reverse transcriptase inhibitor
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRIP	nuclear receptor interacting protein
NtRTI	nucleotide reverse transcriptase inhibitor
OGTT	oral glucose tolerance test
OL	open label
ORO	Oil Red O
OW	overweight
PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline
PC	prospective cohort
PE	streptavidin PE conjugate
PEPCK	phosphoenolpyruvate carboxykinase
PI	protease inhibitor
PIS	prospective intervention study
PI3K	phosphoinositide 3-kinase
PLIN1	perilipin-1
POL	prospective open label
PPARGC	PPAR- γ coactivator
PPAR- γ	peroxisome proliferator-activated receptor gamma
PRT	prospective randomised trial
PUFA	polyunsaturated fatty acid
PPRE	peroxisome proliferator response element
RBL	retinoblastoma-like protein
RCT	randomised controlled trial
RDB	randomised, double blind
RDCr	randomised double blind cross-over
RDPCT	randomised, double blind, placebo-controlled trial
REE	resting energy expenditure
RIT	randomised intervention trial
RNA	ribonucleic acid
RSMCr	randomised single blind controlled multiple crossover
RT	reverse transcriptase
RT-PCR	real-time polymerase chain reaction
RTV	ritonavir
RXR	retinoid-X-receptor

SBP	systolic blood pressure
SC	subcutaneous
SCAT	subcutaneous adipose tissue
SCD	stearoyl-CoA desaturase
SEM	standard error of the mean
SD	standard deviation
SGBS	Simpson Golabi Behmel Syndrome
SMAD	mothers against decapentaplegic homolog
SNP	single nucleotide polymorphism
SREBP	sterol regulatory element binding protein
TBA	thiobarbituric acid
TC	total cholesterol
TDF	tenofovir
TG	triglyceride
TMB	tetramethylbenzidine
TNF- α	tumour necrosis factor-alpha
Tregs	regulatory T cells
TZD	thiazolidinedione
T2DM	type 2 diabetes mellitus
t10,c12	<i>trans</i> -10, <i>cis</i> -12 conjugated linoleic acid
UCP	uncoupling protein
VAT	visceral adipose tissue
VEGF	vascular endothelial growth factor
WAT	white adipose tissue
WHO	World Health Organisation
WHR	waist-to-hip ratio
WC	waist circumference
ZDV	zidovudine
3TC	lamivudine

Chapter 1

Introduction

HIV associated lipodystrophy syndrome (HALS) is a complication of human immunodeficiency virus (HIV) infection and antiretroviral drugs (ARVs) (Galescu et al., 2013). The prevalence of HALS in UK HIV populations and the causal factors associated with it are currently unknown. HALS may occur as a result of antiretroviral drug-induced adipocyte dysfunction, particularly down-regulation of peroxisome proliferator-activated receptor gamma (PPAR- γ) (Caron et al., 2009). Conjugated linoleic acid (CLA) isomers are putative PPAR- γ ligands (Belury et al., 2002). The aim of this thesis was to investigate the contribution of ARVs to adipocyte dysfunction and the role of CLA isomers in mitigating this adipocyte dysfunction *in vitro*, and to explore the prevalence of lipodystrophy in a London-based HIV population, the factors associated with it and the role of CLA supplementation in improving aspects of lipodystrophy *in vivo*.

1.1 Human immunodeficiency virus

In 1981, five cases of *Pneumocystis carinii* pneumonia were presented to the United States Centre for Disease Control in homosexual men who had also presented with decreased CD4+ T cell count resulting in a deficit in cell-mediated immunity (Gottlieb et al., 1981). The men were later found to be suffering from an acquired immune deficiency syndrome (AIDS), caused by a retrovirus named human T-lymphotropic virus type III / lymphadenopathy-associated virus (Sarngadharan et al., 1984), now referred to as HIV.

The latest global prevalence figure for HIV is 35.3 million, with 6,300 new infections per day in 2012, of which ninety-five percent were in individuals from low- and middle-income countries (World Health Organisation, 2014a). In 2013, over two thousand new cases of HIV were diagnosed in the UK, which brings the total number of new diagnoses to 131,327 in over two decades (Public Health England, 2014). The UK HIV population represents less than one percent of the HIV/AIDS burden worldwide, while sixty-eight percent of the global HIV/AIDS population is located in Sub-Saharan Africa (World Health Organisation, 2010). Since 2005, mortality from HIV/AIDS has declined by 30%, which was accompanied by and partly attributed to a 24-fold increase in the number of people receiving treatment with ARVs (World Health Organisation, 2014b).

There are currently twenty-four ARVs from five different drug classes licensed for use in the European Union (EU; **Table 1.1**). Drugs within each ARV class differ in their mode of action and effects on the stages of HIV replication (**Figure 1.1**). Nucleoside reverse transcriptase inhibitors (NRTIs) interact with the substrate binding site of HIV reverse transcriptase (RT) (Kakuda, 2000). Nucleotide reverse transcriptase inhibitors (NtRTIs) require only two steps of phosphorylation, as opposed to three with the NRTIs, but work in a similar way to NRTIs (Gilead Sciences Inc, 2001). Non-nucleoside reverse transcriptase inhibitors (NNRTIs) differ from NRTIs in that they bind specifically with a non-substrate binding site of the RT enzyme, disrupting the enzymes catalytic site. Protease inhibitors (PIs) inhibit the protease enzyme, thus preventing the cell from cleaving the proteins into active viral particles (Wynn et al., 2004). Fusion inhibitors block the attachment, co-receptor binding and fusion of the viral particle and prevent viral capsid entry into the host cell (Greenberg and Cammack, 2004). C-C chemokine receptor type 5 (CCR5) inhibitors inhibit CCR5 signalling, are antagonists of the CCR5 receptor and prevent viral entry into the host cell (Dorr et al., 2005). Integrase inhibitors are the newest class of ARVs and work by inhibiting the insertion of the HIV-1 pro-viral DNA into the host cell genome (De Clercq, 2010).

Table 1.1 Antiretroviral drugs currently licensed in the EU

Generic name	Trade name
Fixed dose combinations	
Lamivudine (3TC) / abacavir	Kivexa
3TC/ zidovudine (ZDV)	Combivir
Emtricitabine (FTC)/ tenofovir	Truvada
3TC/abacavir/ ZDV	Trizivir
FTC/rilpivirine/ tenofovir	Eviplera
FTC/ tenofovir/ efavirenz	Atripla
Elvitegravir/ cobicistat/ FTC/ tenofovir	Stribild
NRTIs	
3TC	Epivir
Abacavir	Ziagen
ZDV	Retrovir
FTC	Emtriva
NtRTIs	
Tenofovir	Viread
NNRTIs	
Efavirenz	Sustiva
Etravirine	Intelence
Nevirapine	Viramune
Rilpivirine	Edurant
PIs	
Atazanavir	Reyataz
Darunavir	Prezista
Fosamprenavir	Telzir
Lopinavir/ritonavir	Kaletra
Ritonavir	Norvir
Tipranavir	Aptivus
CCR5 inhibitor	
Maraviroc	Celsentri
Integrase inhibitor	
Raltegravir	Isentress

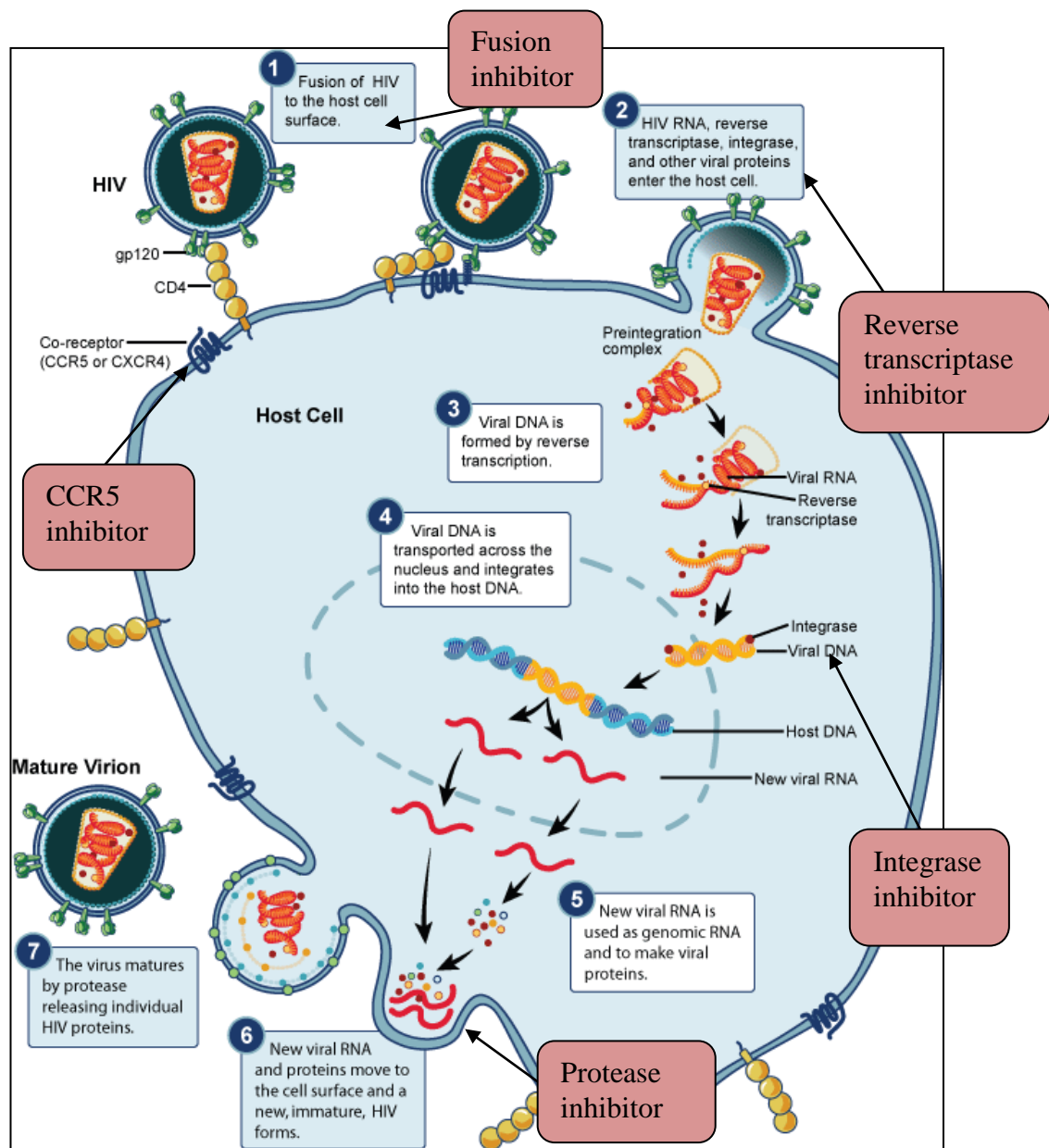


Figure 1.1 Mode of action of antiretroviral drugs (Adapted from (National Institute of Allergy and Infectious Diseases, 2010).

In 1987, zidovudine (ZDV) became the first ARV approved by the Food and Drug Administration (FDA) for the management of HIV/AIDS (Food and Drug Administration, 2011). The development of successive ARVs would give rise to the first combination with ZDV and dideoxycytidine (McLeod and Hammer, 1992). Combination drug therapy, also known as highly active antiretroviral therapy (HAART), consists of at least two ARVs, most commonly NRTIs, PIs and NNRTIs (Shafer and Vuitton, 1999). For those with access to ARVs, HIV infection has been transformed into a manageable, chronic condition. HAART introduction has led to a significant reduction in mortality among people living with HIV/AIDS (Palella et al., 1998). However, the beneficial impact of HAART is marred to some extent by the adverse effects associated with its use. HIV infection and HAART are associated with the development of adverse neurological, gastrointestinal, hepatobiliary, respiratory, endocrine, reproductive, renal, skeletal and cardiovascular (CV) / metabolic effects (**Figure 1.2**). The focus of this thesis is the cardiometabolic effects of HAART and HIV.

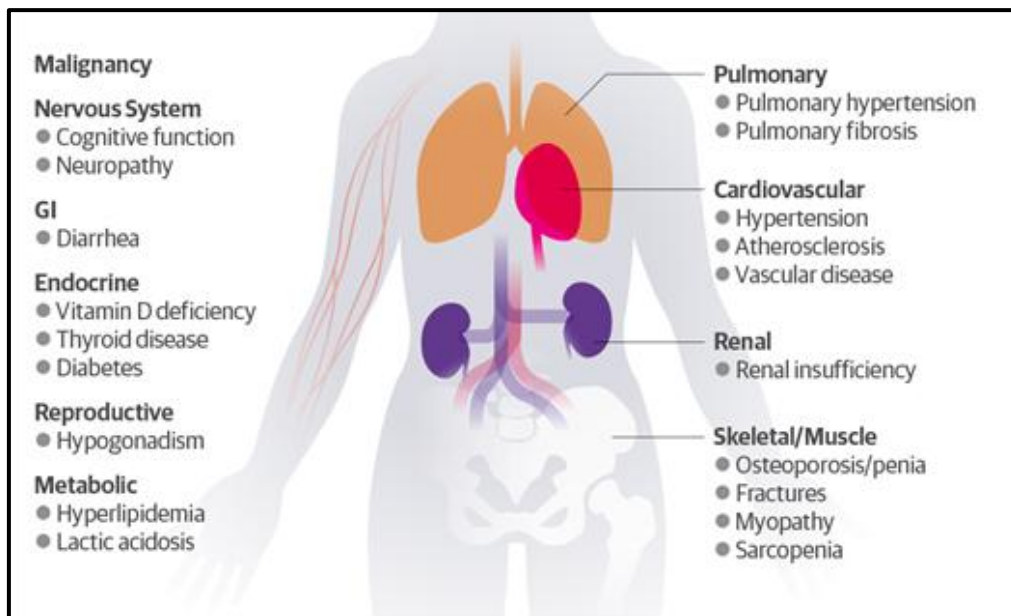


Figure 1.2 Spectrum of HIV complications (*Adapted from (NAM., 2012)*)

1.2 HIV-associated lipodystrophy syndrome

HALS is one of the complications of HAART and was first described by Carr and colleagues in 1998 (Carr et al., 1998a). HALS has since become the most prevalent subtype of acquired lipodystrophy (LD) (Garg, 2011). HALS is characterised by lipoatrophy (LA) in the face and extremities, lipohypertrophy (LH) in the abdomen and dorsocervical region, accompanied by dyslipidaemia, insulin resistance (IR), impaired glucose tolerance, hypertension (HTN), endothelial dysfunction, and altered cytokine and adipokine production (Galescu et al., 2013). A recent systematic review and meta-analysis found that HIV-infected individuals receiving HAART had a two-fold increased risk of cardiovascular disease (CVD) compared with healthy individuals (Islam et al., 2012). An increased risk of acute myocardial infarction has also been shown in HIV-infected individuals receiving ARVs, particularly the PI ritonavir (RTV), which was associated with a greater than two-fold increased risk (Durand et al., 2011). Though ARVs, particularly PIs and NRTIs, are considered the main drivers of HALS, the virus itself (Agarwal et al., 2013) and host genetics (Marzocchetti et al., 2011), may also contribute to its pathogenesis.

1.2.1 Clinical characteristics of HALS

1.2.1.1 Lipoatrophy

LA in HIV is characterised by loss of subcutaneous (SC) fat (Carr et al., 1998a) and is distinctly different from traditional HIV wasting, which is characterised by a decrease in lean body mass (Grinspoon et al., 1996). LA is seen mainly in the face (facial LA), arms, legs and buttocks (peripheral LA) (Saint-Marc et al., 2000, Lichtenstein et al., 2003). Facial LA usually presents as malar or temporal wasting (Omolayo, 2008). Peripheral LA is often accompanied by prominent superficial veins, which contribute to the emaciated appearance observed in these individuals (Bergersen et al., 2005). It has been shown that at least 30% of limb fat needs to be lost before LA becomes clinically evident (Podzamczar et al., 2009). Although LA was originally attributed to PIs (Carr et al., 1998a), a recent systematic review concluded that NRTIs are the main contributor (de Waal et al., 2013). Older NRTIs such as stavudine (d4T) are associated with a significantly greater rate of limb fat loss in HIV-infected men compared with other drugs (Mallon et al., 2003). The World Health Organisation (WHO) now recommends ZDV- or tenofovir (TDF)-based first line regimens in favour of d4T due to the long

term toxicities associated with d4T which include LA (World Health Organisation, 2013). Other risk factors associated with LA include older age, a decrease in body mass index (BMI) of $\geq 2 \text{ kg/m}^2$, white race, low recent CD4 cell count percentage, low nadir CD4 count or percentage, and a diagnosis of AIDS (Lichtenstein et al., 2001). Generally, morphological alterations associated with HALS including LA tend to occur later in the course of treatment. One study showed that patients treated with a NRTI or a PI for less than three months did not develop LA (Ho et al., 1999). In accordance with these findings, Carr *et al* estimated the mean time to developing LD as ten months after commencing PI therapy (Carr et al., 1999). A recent study found that 28% of a cohort of 2,131 patients developed LA after 1-5 years of receiving HAART (Leclercq et al., 2013).

1.2.1.2 Lipohypertrophy

LH is characterised by adipose tissue accumulation mainly in the intra-abdominal (Carr et al., 1998a, Dinges et al., 2005) and dorsocervical regions (Lo et al., 1998, Roth et al., 1998). Other features of LH may include breast enlargement, observed in both males and females (Bernasconi et al., 2002, Savès et al., 2002, Mutimura et al., 2007), accumulation of adipose tissue on the anterior region of the neck (Palella et al., 2006), side of the neck (Mutimura et al., 2007), under the axillae (Palella et al., 2006) and in the suprapubic region (Guaraldi et al., 2007), and localised or generalised lipomas (Miller et al., 2003). LH in the context of HIV/HAART is distinct from simple accumulation of visceral adipose tissue (VAT), as it is associated with a decrease, rather than an increase, in subcutaneous adipose tissue (Dinges et al., 2005). Interestingly, central obesity is the most commonly identified form of LH seen among HALS patients (Loonam and Mullen, 2012).

Factors associated with the development of LH in the context of HIV and HAART include being female (Jacobson DL et al., 2005), older age, white race, type 2 diabetes mellitus (T2DM) and CD4 count > 300 (Nguyen et al., 2008), CD8 cell activation (Guaraldi et al., 2013), total cholesterol (TC) $\geq 5 \text{ mmol/L}$ and triglycerides (TG) $\geq 1.75 \text{ mmol/L}$ (Nguyen et al., 2008). PIs (indinavir (IDV), abacavir, nelfinavir) and NNRTIs (efavirenz) have also been shown to be associated with LH (Nguyen et al., 2008). Interestingly, greater levels of VAT and a higher prevalence of metabolic syndrome (MetS), T2DM and CVD risk have been found in men with a high TG/high waist circumference (WC) ratio (Janiszewski et al., 2011). Recently, it has been observed that

central obesity in HIV-infected individuals is associated with physical frailty (Shah et al., 2012) and neurocognitive impairment possibly as a result of cerebral degeneration associated with inflammation and IR (McCutchan et al., 2012).

1.2.1.3 Dyslipidaemia

The incidence of dyslipidaemia is higher in HIV-infected individuals receiving HAART compared with treatment-naïve individuals and non-HIV-infected individuals (Tripathi et al., 2013). The prevalence of dyslipidaemia in HIV has been shown to vary from 24% to 72% (Loonam and Mullen, 2012). Dyslipidaemia in HIV-infected individuals arises as a result of HIV infection, as well as treatment with HAART (Grunfeld et al., 1989, Pinto Neto et al., 2013). HAART is associated with the development of an atherogenic lipid profile including elevated TC, low-density lipoprotein cholesterol (LDL-C) and TG (Duro et al., 2013). Interestingly, the same researchers found increased levels of high density lipoprotein cholesterol (HDL-C) in patients treated for 3 years with an NNRTI containing regimen (Duro et al., 2013). This protective effect of NNRTI on HDL-C has also been demonstrated in other studies (van der Valk et al., 2001, Friis-Moller et al., 2003). Data from HIV negative healthcare workers treated prophylactically with HAART following sharps injury showed elevated TC and LDL-C after only four weeks of treatment (Allan and Behrman, 2001). Although one study found no effect of HAART duration on risk of dyslipidaemia (Duro et al., 2013), data from large cohort studies (Friis-Moller et al., 2003, Young et al., 2005, Domingo et al., 2008), cross-sectional studies (Ceccato et al., 2011, Feleke et al., 2012) and prospective studies (Heath et al., 2001, Heath et al., 2002) demonstrate an association between increasing duration of HAART and risk of dyslipidaemia. Age and sex (Leitner et al., 2006), race (Foulkes et al., 2006) and genetic factors (Egana-Gorrone et al., 2013) have also been shown to be associated with risk of dyslipidaemia in HIV.

Dyslipidaemia in HIV-infected individuals may be diagnosed using recommendations for the general population, i.e. using the National Cholesterol Education Programme Adult Treatment Panel III (NCEP) guidelines (Gkrania-Klotsas and Klotsas, 2007). The authors of this paper recommended that fasting lipid profile tests be offered to patients before commencing HAART in order to monitor any changes caused thereafter.

1.2.1.4 Atherosclerosis

Atherosclerosis is characterised by the formation of plaques in large- and medium-sized arteries and is an initial step in the development of CVD (Gibellini et al., 2013). Atherosclerotic plaques form within the sub-endothelial intima structure when LDL and lipoprotein remnants become trapped, are modified and then activate the endothelium. The activated endothelium and modified LDL induce chronic inflammation and are associated with an increase in monocyte recruitment and differentiation to macrophages. In the presence of chronic inflammation, activated macrophages produce cytokines, chemokines and proteases, which lead to the formation of foam cells and migration of smooth muscle cells into the endothelial intima. As the atherosclerotic plaque increases in size, it induces vascular damage and eventually, rupture of the plaque and thrombosis may lead to a coronary event (Gibellini et al., 2013). Atherosclerotic plaques, by definition, are “lesions of the intima characterised by necrotic and calcified regions containing accumulated, modified lipids, inflamed smooth muscle cells, leukocytes and foam cells” (Gibellini et al., 2013).

HIV-infected men have a higher prevalence of subclinical coronary atherosclerosis compared with non-infected men with similar demographics and risk factors for heart disease (59% vs. 34%) (Lo et al., 2010). LD was found to predict endothelial dysfunction (Masiá, 2010), which is a critical step in the progression of atherosclerosis in HIV (Shankar and Dube, 2004). Data from the AIDS Clinical Trials Group show a rapid improvement in endothelial function in previously naive patients randomised to treatment (Torriani et al., 2008). Similarly, a study in patients receiving HAART found no association between HAART and endothelial function measured using flow-mediated dilatation (Dubé et al., 2010). Contrary to this, a study of 37 adults receiving HAART showed increased endothelial dysfunction in those taking PIs compared to those who were not (Stein et al., 2001). Atherosclerosis progression, as measured by carotid intima media thickness, was found to be increased as a result of HAART (van Vonderen et al., 2009, Vigano et al., 2010). Other factors associated with atherosclerosis in HIV include TC, LDL-C and longer duration of HAART, which predict plaque burden (Lo et al., 2010), lower nadir CD4 count which is associated with coronary stenosis of greater than 50% (Post et al., 2014) and long term HIV infection, which independently predicts early carotid atherosclerosis (Lorenz et al., 2008).

1.2.1.5 Endothelial dysfunction

Endothelial dysfunction is one of the proposed underlying mechanisms involved in the development of CVD in HIV (López et al., 2012). Untreated HIV infection has been associated with greater endothelial dysfunction (Arildsen et al., 2013). Brachial artery flow mediated dilatation (FMD) provides a measure of blood flow through the blood vessel, as well as vessel dilation, and has been shown to be normalised upon initiation of HAART in HIV-infected individuals with low FMD readings compared with controls (Arildsen et al., 2013). A recent study showed a decrease in biomarkers for endothelial damage in treatment-naïve patients with HIV after 12 weeks of HAART (Mata-Marín et al., 2013). Type of HAART has been shown to play a role, as was shown in one study where worsening endothelial function was observed in patients treated with the NNRTI efavirenz compared with PIs at 12 months (Gupta et al., 2012). HAART duration, associated with a higher circulating lipopolysaccharide (LPS) concentration, was also shown to be a predictor of FMD in patients with HIV (Blodget et al., 2012). In a study of ninety-six HIV-infected individuals, no association was found between endothelial function assessed by brachial FMD and any body composition measure, HAART use, PI use, CD4 cell count, and HIV RNA levels (Dubé et al., 2010). Although this study failed to find an association for PI use *in vivo*, work in human aortic endothelial cells has shown PI to mediate endothelial dysfunction, which was ameliorated by the addition of troglitazone, an anti-diabetic drug and potent PPAR- γ agonist (Mondal et al., 2013).

1.2.1.6 Hypertension

HTN is a major modifiable risk factor for CVD and is prevalent in up to 43% of patients with HIV (Myerson et al., 2014). In some studies, rates of 30%, similar to the general population (Joffres et al., 2013), have been reported (De Socio et al., 2014). In a cohort of London-based HIV patients, raised systolic blood pressure (SBP) was identified in 18% of patients (Elgalib et al., 2011). HTN in HIV is associated with both LA and LH (Crane et al., 2009). Patients classified as having isolated LH and mixed forms of LD were shown to have a higher prevalence of HTN compared with those with LA and with no LD. HTN associated with LH is suggested to be mediated by increased BMI (Crane et al., 2009).

A recent, multicentre, cross-sectional study of 1,182 HIV patients found a number of factors to be predictive of HTN including age ≥ 50 years, male sex, BMI ≥ 25 kg/m², central obesity, family history of CVD, previous CV events, T2DM and MetS, as well

as duration of HIV infection, duration of HAART, and low nadir CD4 cell count (De Socio et al., 2014). Contrary to these findings, Elgalib *et al* (2011) found that higher CD4 cell count was associated with raised SBP in their cohort of 1,022 London-based patients. The role of HAART in mediating HTN is unclear as some studies associate HTN with longer duration of HAART (De Socio et al., 2014), while others found no association between HAART and HTN (Thiébaud et al., 2005, Medina-Torne et al., 2012) and use of some NNRTI was found to lower the risk of HTN (Thiébaud et al., 2005). Recently, alcohol abuse has been independently associated with HTN in patients with HIV (Ikeda et al., 2013).

1.2.1.7 Cardiovascular disease

As discussed in previous sections, both HIV and HAART alter the profile of traditional CV risk factors (see **Figure 1.3**) and increase the risk of CVD in HIV-infected individuals (Bergersen et al., 2004). The role of viraemia in CVD was demonstrated by El-Sadr *et al* (2008) who found that patients randomised to episodic use of HAART based on CD4 count had higher rates of CVD compared with patients with uninterrupted HAART who maintained viral suppression. With respect to ART, increased CVD risk was found to be associated with each additional year of exposure to PIs (IDV and lopinavir) (Friis-Moller et al., 2007, Bavinger et al., 2013) and NRTIs (abacavir) (Bavinger et al., 2013), but not NNRTIs (Friis-Moller et al., 2007), newer generation PIs, CCR5 antagonists, or integrase inhibitors (Longenecker and Triant, 2014). Data from a London-based cohort show an association between duration of first line HAART and a coronary heart disease (CHD) risk of $\geq 10\%$ (Elgalib et al., 2011). However, the role of HAART in mediating CVD in HIV was recently reviewed and findings suggest that use of HAART, even at higher CD4 counts, may reduce CVD risk (Longenecker and Triant, 2014). It is important to note that this was not a systematic review.

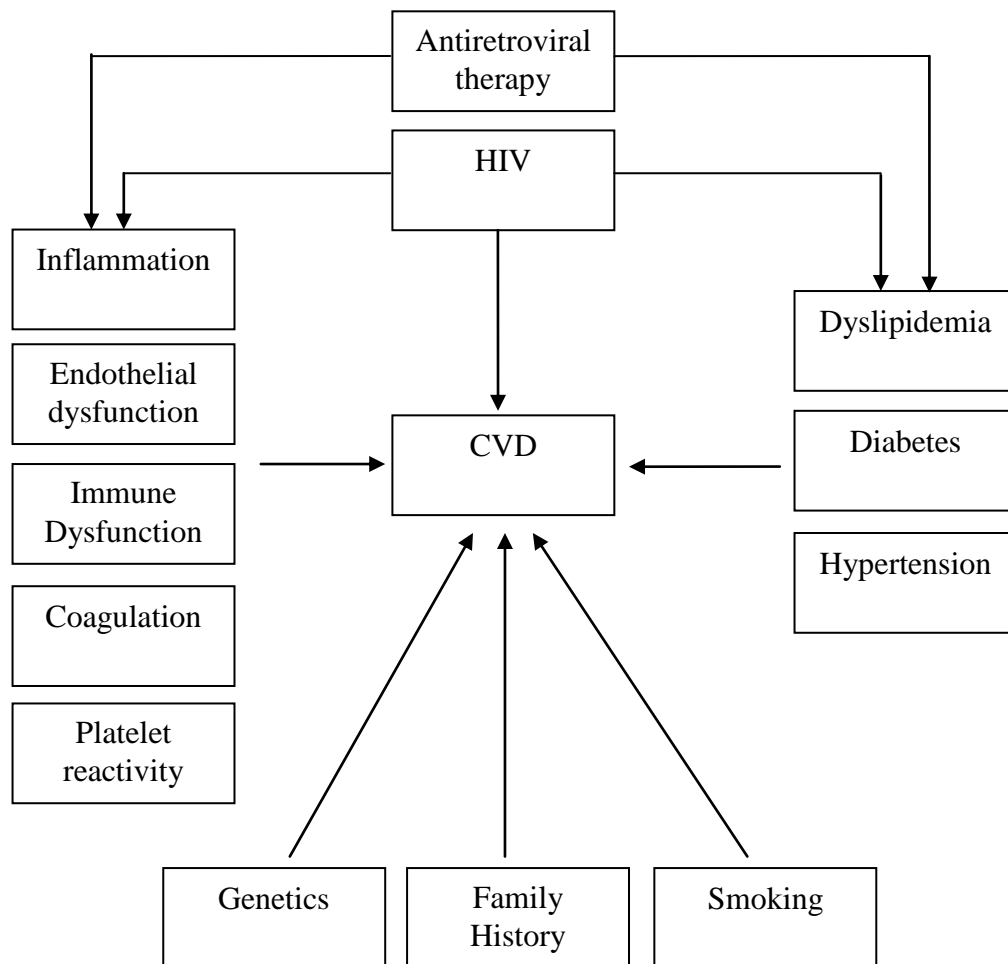


Figure 1.3 CVD risk factors in HIV (*Reproduced from (Clinical Care Options, 2010)*)

The risk of CV events increases in patients with MetS compared with those without (22.2% vs. 7.4%) (Alvarez et al., 2010). However, family history has been shown to be a greater predictor of CVD risk than T2DM, HTN and smoking in HIV-infected individuals (Calvo-Sánchez et al., 2013). Ten-year CVD risk is frequently estimated using the Framingham equations; however, the Data collection on Adverse events of Anti-HIV Drug study equation is the only tool specifically designed to predict CVD risk in HIV-infected populations (Nery et al., 2013). The PROCAM study equation is also used to predict 10-year CVD risk, but, again, is not specific for HIV-infected individuals (Nery et al., 2013). Using the Framingham equations, a London-based study of HIV positive adults identified an average of 6% risk of CVD with a median risk of 4% in men and 1.4% in women. Caucasians had a three-fold increase in the prevalence of CVD risk > 20% compared with other ethnicities and raised TC was the greatest risk factor for CVD (Aboud et al., 2010).

Traditional risk factors cannot fully explain the increased risk of CVD in HIV. The role of immune activation and inflammation in the pathogenesis of CVD in HIV has recently been reviewed (Triant, 2013). Acute phase proteins such as IL-6 and C-reactive protein (CRP), as well as biomarkers of coagulation (D-dimer) are associated with CVD risk factors in HIV (Duprez et al., 2012, Hileman et al., 2014). Treated HIV-infected patients demonstrate increased monocyte expression of pro-inflammatory IL-1 β compared with uninfected individuals (19.5% vs. 1.9%), which is predictive of high plasma IL-6 (Jalbert et al., 2013). The presence of obesity in patients with HIV has an additive effect on inflammatory biomarkers resulting in higher serum IL-6 and macrophage inflammatory protein 1- α compared with non-obese patients (Koethe et al., 2013). Recent evidence indicates that despite virological suppression in patients receiving HAART, markers of inflammation and immune activation persist, which may contribute to chronic inflammation (Reingold et al., 2008, French et al., 2009). Moreover, a higher prevalence of atherosclerosis (78% vs. 42%) and immune activation has been demonstrated among elite controllers (controlled HIV infection in the absence of therapy) compared with uninfected controls (Pereyra et al., 2012). Both of these factors argue for an effect of HIV disease parameters in the pathogenesis of CVD.

1.2.1.8 Altered adipokine and cytokine expression

Altered expression and secretion of adipokines is a characteristic feature of HALS (Paruthi et al., 2013). Adiponectin, leptin, resistin and interleukin-6 (IL-6) are some of the main adipokines affected in HALS. Adiponectin is a relatively abundant plasma adipokine with anti-inflammatory actions involved in the regulation of glucose metabolism and peripheral insulin sensitivity (Ouchi et al., 2011). Adiponectin concentration in HALS was found to be 40% lower than in healthy controls and 43% lower than HIV-infected individuals without HALS (Bezante et al., 2009). This finding of hypoadiponectinaemia in HALS has been demonstrated by a number of groups (Lindegard et al., 2004, Samaras et al., 2007, Luo et al., 2009, Veloso et al., 2012). Adiponectin levels in HALS correlate negatively with glucose, IR and TG (Veloso et al., 2012) and have been associated with current or previous d4T use (Lindegard et al., 2004). Pro-inflammatory adipokines leptin, resistin and IL-6 are significantly altered in HALS, which may contribute to inflammation, a key step in the pathogenesis of HALS (Caron-Debarle et al., 2010a). Furthermore, recent evidence points towards an association between inflammation in HIV and declines in functional performance as determined by hand grip strength (Jalbert et al., 2013).

Leptin is an important regulator of lipid metabolism, insulin action and energy homeostasis (Ouchi et al., 2011). Circulating plasma leptin reflects adiposity in HIV, where patients with LA have lower leptin levels (1.76 ng/ml) compared with patients with LH (9.10 ng/ml) and those without LD (3.14 ng/ml) (Nagy et al., 2003). Leptin replacement therapy was found to improve dyslipidaemia (Mulligan et al., 2009) and glycaemia (Sekhar et al., 2012) in patients with LA suggesting a role for hypoleptinaemia in the pathogenesis of these abnormalities in HALS.

Resistin is another pro-inflammatory adipokine, originally suggested to be the link between obesity and IR (Steppan et al., 2001). A recent cross-sectional study of 100 patients found that LA was associated with a 20% increase in resistin compared with patients without HALS (Arama et al., 2014). In contrast, no association was found between serum resistin levels and LA, LH or mixed syndrome in a cross-sectional study of 227 patients (Barb et al 2005). Differences might be explained by the way in which HALS was defined in each study; Barb *et al* used three blinded investigators to determine fat distribution based on measured body composition, while Arama *et al* did not measure body fat and relied on a questionnaire to identify the presence or absence of features of HALS. Plasma resistin levels have been shown to correlate with IR in HALS (Escote et al., 2011) and single nucleotide polymorphisms (SNPs) in the resistin gene have been found to be associated with HALS (Ranade et al., 2008, Escote et al., 2011). Both of these factors indicate the potential implication of resistin in HALS, although its exact role has yet to be determined.

Adipose tissue produces a significant proportion of circulating IL-6, which acts as a pro-inflammatory cytokine and is thought to link obesity-associated inflammation with IR (Ouchi et al., 2011). Plasma IL-6 has been shown to correlate with IR in HALS (Vigouroux et al., 2003) and serum IL-6 concentrations were increased in HALS compared with subjects without HALS and healthy controls (Johnson et al., 2004). Other studies have shown no difference in plasma IL-6 between those with HALS and those without (Kannisto et al., 2003, Saumoy et al., 2008). In subcutaneous adipose tissue (SCAT) biopsy samples from HALS patients, IL-6 mRNA expression was found to be significantly higher compared with patients without HALS (Kannisto et al., 2003) and healthy controls (Jan et al., 2004). Another study found no difference in SCAT expression of IL6 in patients with HALS compared to healthy controls, but

interestingly, IL-6 expression in VAT was significantly decreased (Gallego-Escuredo et al., 2013).

1.2.1.9 Insulin resistance and type 2 diabetes mellitus

T2DM in HIV-infected individuals was relatively uncommon prior to the antiretroviral therapy (ART) era (Bradbury and Samaras, 2008). Following the introduction of PIs, a greater number of reported glucose disorders began to emerge in HIV infected individuals (Dubé et al., 1997, Eastone and Decker, 1997, Visnargarwala et al., 1997). In a recent study, patients with LD (defined by fat mass ratio) had significantly higher plasma glucose, insulin, HbA1c and IR compared with those without LD (Freitas et al., 2012). Accordingly, patients with LD had a higher prevalence of impaired fasting glucose (15.7% vs. 5%), impaired glucose tolerance (21.4% vs. 19%) and T2DM (21.4% vs. 14%) than patients without LD. Impaired glucose tolerance was also more prevalent among patients with abdominal LD in this study, compared with those without a high WC (30.9% vs. 16.2%). IR has been identified in up to 66 % of HIV-infected patients stable on HAART (Arama et al., 2013). Another study compared 6816 HIV-infected individuals with the same number of non-HIV infected controls and found a higher incidence of T2DM among non-HIV infected individuals compared with infected (13.6 vs. 11.4 per 1000 person-years) (Tripathi et al., 2014). Risk factors for T2DM in HIV have been found to include older age, female gender, non-white race/ethnicity, higher BMI, higher TG, lower TC, pre-existing HTN, hepatitis C virus (HCV) infection, longer duration of HIV infection and lower nadir CD4 (Galli et al., 2012, Tripathi et al., 2014).

Diagnosis of glucose disorders in HALS has been made on the basis of guidelines from the International Diabetes Federation (IDF) (International Diabetes Federation, 2005) and the American Diabetes Association (American Diabetes Association and Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003). According to these guidelines, impaired glucose tolerance is defined as a fasting glucose (FG) of greater than 5.6 mmol/L and a value of greater than 7 mmol/L is indicative of diabetes. Recent European AIDS Clinical Society guidelines recommend testing patients for FG at HIV diagnosis, before the start of HAART and annually thereafter (Lundgren et al., 2008). Society guidelines for diagnosing diabetes and its pre-stages are based on those from the WHO 2006 and the IDF (**Table 1.2**) (World Health Organisation and International Diabetes Federation, 2006).

Table 1.2 Diagnosis of diabetes and its pre-stages

Diabetes**Any one of the following:**

FG ≥ 7 mmol/L2-hour plasma glucose ≥ 11.1 mmol/L75 g OGTT with FG ≥ 7 mmol/LGlycated haemoglobin (HbA1c) $\geq 6.5\%$ Random plasma glucose ≥ 11.1 mmol/L in the presence of classical diabetes symptoms

Impaired fasting glucose

FG 6.1 – 6.9 mmol/L **and if measured**2-hour plasma glucose < 7.8 mmol/L

Impaired glucose tolerance

FG < 7 mmol/L **and**2-hour plasma glucose ≥ 7.8 mmol/L and < 11.1 mmol/L

OGTT, oral glucose tolerance test. FG, fasting glucose.

1.2.1.10 Energy dysregulation

Energy dysregulation is another characteristic of HALS and has been investigated in a few studies, the results of which are summarised in **Table 1.3**. A meta-analysis of studies investigating energy expenditure in HIV found that resting energy expenditure per kg lean body mass (REE/LBM) was significantly higher in HIV-infected individuals compared with healthy controls, particularly for symptomatic individuals (Batterham, 2005).

Table 1.3 Energy expenditure in HALS

Reference	Subjects	Change in energy expenditure
Kosmiski <i>et al</i> (2001)	PI-treated, with HALS	14.6% and 32.5% increased REE compared with PI-treated without HALS and PI-naive without HALS, respectively.
Kosmiski <i>et al</i> (2003b)	PI-treated, with HALS	67.8 % and 56.4% increased REE compared with HIV positive without HALS and healthy subjects, respectively.
Kosmiski <i>et al</i> (2007b)	PI-treated, with HALS	15% increased REE compared with healthy subjects
Sutinen & Yki-Jarvinen (2007)	HAART-treated with HALS	11% increased REE compared with HIV+ subjects without HALS

PI, protease inhibitors. REE, resting energy expenditure. HALS, HIV-associated lipodystrophy syndrome. HAART, highly active antiretroviral therapy.

Sutinen and Yki-Jarvinen (2007) showed that the increased energy expenditure found in their subjects with HALS was accompanied by an 18.6% higher total caloric intake compared with those without HALS (2,289 kcal/d vs. 1,861 kcal/d). In addition to increased REE, studies by Kosmiski *et al* have shown that 3 days of eu-energetic feeding (energy requirements = REE x activity factor of 1.3), which should not induce a change in REE, resulted in a significantly higher REE among HIV positive adults with LD compared with healthy controls (33 vs. 28 kcal/kg lean body mass (LBM) (Kosmiski *et al.*, 2007b). The same researchers found that three days of hypo-energetic feeding (energy requirements = 50% less than eu-energetic period) induced a 7.5% reduction in REE and three days of hyper-energetic feeding (energy requirements = 50% more than eu-energetic period) caused a 4.3% increase in REE in HIV positive adults with LD but not in patients without LD or healthy controls (Kosmiski *et al.*, 2007a). The group concluded that individuals with LD have higher REE per kg LBM than those without LD, short-term caloric restriction reduces REE and short-term over-feeding increases REE in those with LD. The authors suggest that hypermetabolism associated with LD and a form of adaptive thermogenesis invoked to dissipate calories that cannot be stored in a normal manner underlie these observations. Data from the Nutrition for Healthy Living study also show increased REE in individuals receiving HAART compared to those not receiving HAART (Mangili *et al.*, 2006), while

Vassimon *et al* (2012) demonstrated a 22% and 56% increased fasting carbohydrate oxidation in HALS compared with men without HALS and healthy controls, respectively. Additionally, lipid oxidation in men with HALS was 23% and 53% lower in HALS compared with men without HALS and healthy controls, respectively. A recent study showed a 2% increase in REE in HAART-naïve women, which remained elevated when on HAART, suggesting a role for the virus itself in instigating changes in REE (Mittelsteadt *et al.*, 2013).

1.2.2 Defining HALS

It is almost two decades since HALS was first described (Carr *et al.*, 1998a) and a precise definition for the syndrome has yet to be established. Carter *et al.* (2001) showed that the prevalence of HALS can vary between 19% - 65% as a result of differences in the definition of HALS. Furthermore, LA and LH have been shown to occur together as well as separately which has led to much confusion regarding the definition of the syndrome. Existing definitions for the syndrome include LA or LH, LA alone, LH alone or a combination of LA and LH. A recent systematic review concluded that LA is a consequence of HAART, but LH is not as it occurs to the same degree in those receiving different HAART regimens, is not linked to any specific drugs, and can be improved by changes to lifestyle (de Waal *et al.*, 2013). The authors of this study suggest that LH occurs only as a result of controlling HIV infection, which normalises the inflammatory profile.

The main definitions used for the metabolic alterations associated with HALS include the NCEP guidelines (National Cholesterol Education Program Expert Panel on Detection Evaluation and Treatment of High Blood Cholesterol in Adults, 2002) (Worm *et al.*, 2010), the IDF Guidelines (International Diabetes Federation, 2005) (Zannou *et al.*, 2009), a combination of NCEP and IDF (Samaras *et al.*, 2007), or the “Report of the National Heart, Lung, and Blood Institute/ American Heart Association Conference on Scientific Issues Related to Definition” (Grundey *et al.*, 2004) (Jacobson *et al.*, 2006).

In a recent cohort study of more than 34,000 subjects from the general European population, the prevalence of the MetS was found to be 24.3% (Scuteri *et al.*, 2014). Although the exact prevalence is unclear, prevalence of MetS in international HIV populations ranges from 11-45% (Paula *et al.*, 2013). HIV-infected individuals with

HALS display features of the MetS including abdominal obesity, dyslipidaemia, IR, HTN, endothelial dysfunction, and a proinflammatory and prothrombotic state (International Diabetes Federation, 2006). However, unlike MetS in the general population, abdominal obesity in HALS may also occur alongside LA.

Self-report and physician examinations are frequently used to diagnose HALS; however, these definitions have been shown to vary and the accuracy of these subjective methods is unclear (Loonam and Mullen, 2012). In addition to patient self-report and physician reports, anthropometric techniques such as skinfold thickness measurements are also used to identify body fat changes in HIV (Siqueira Vasimon et al., 2011). Skinfold measurements have been used to calculate fat mass ratio (FMR; ratio of the percentage of trunk fat mass to the percentage of the lower limb fat mass) in HIV and have been shown to be particularly useful among HIV-infected individuals in resource poor settings (Asha et al., 2011). Bioelectrical impedance analysis (BIA) is used to measure body composition in HIV, and is useful in measuring total fat mass (Freitas et al., 2011). BIA has been shown to be more precise than skinfold measurements, can be repeated and is a non-invasive way of determining total fat mass (Siqueira Vasimon et al., 2011, Perez-Matute et al., 2013). Dual energy x-ray absorptiometry (DXA) was used early in the post-HAART era to determine body composition (Grinspoon et al., 1999) and over a decade later, continues to be used to evaluate fat mass in HIV (Freitas et al., 2012). Computed tomography (Freitas et al., 2012) and whole body magnetic resonance imaging (Lake et al., 2011) have been used to investigate abnormalities of body composition in HIV-infected individuals. Although these measures provide accurate body composition data, the cost associated with their use negates their use in routine clinical practice. Sonography has been used by some researchers to define HALS (Signorini et al., 2012) and has been shown to be accurate in measuring intra-abdominal fat, waist-to-hip ratio (WHR) and subcutaneous leg fat (Padilla et al., 2007). However, the same researchers showed that skinfold measurements and WHR had greater reproducibility than ultrasonography.

In 2003, Carr and colleagues proposed an objective definition for HALS based on age, sex, duration of HIV infection, HIV disease stage, WHR, serum anion gap, HDL-C concentration, trunk to peripheral fat ratio, percentage leg fat, and intra-abdominal to extra abdominal fat ratio (Carr, 2003). **Table 1.4** shows the case definition and scoring system used. A diagnosis of LD is made by deriving a total LD score by adding the

individual scores for each variable, and then subtracting a constant value of 43. If the final score is at least zero or greater then the patient can be diagnosed with LD, while a score of less than zero indicates no LD. This definition requires anthropometric variables from DXA and CT, which reduces its utility in clinical practice. Other research groups have also determined fat mass distribution objectively by creating standard values for a fat mass ratio (FMR; ratio of the percentage of the trunk fat mass to the percentage of the lower limb fat mass) in uninfected men and applying it to HIV-infected men (Bonnet et al., 2005). Using variables from DXA another group defined gender-specific FMR cut offs as 1.961 for men and 1.329 for women, which provide another objective method of defining HALS (Freitas et al., 2010).

Table 1.4 HALS objective definition and scoring system

Factor	Score
Demographic	
Sex	
Male	0
Female	22
Age	
< 40 years	0
> 40 years	7
Duration HIV	
< 4 years	0
> 4 years	11
CDC HIV stage	
A	0
B	3
C	7
Clinical	
WHR (0.1)	Multiply by 29
Metabolic	
HDL-C (0.1 mmol/L)	Multiply by -14
Anion gap (1 mmol/L)	Multiply by 1
Body composition	
VAT/SCAT ratio	
< 0.45	0
0.45-0.83	-2
0.83-1.59	3
> 1.59	13
Trunk/limb fat ratio (1.0)	Multiply by 5
Leg fat (%)	
> 21.4	-16
14.5-21.4	-14
8.8-14.5	-8
< 8.8	0

(Adapted from Carr et al 2003)

1.2.3 Molecular pathogenesis of HALS

One of the first proposed mechanisms underpinning HALS was “abnormal lipid release or storage perhaps via adipocyte apoptosis” (Carr et al., 1998b). Since then, a definitive association between disturbances in adipose tissue metabolism and HALS has been established including altered adipocyte differentiation, insulin sensitivity, adipokine secretion and mitochondrial toxicity (Giralt et al., 2011). Both PIs and NRTIs have been shown to cause disturbances in adipocyte function (Stankov et al., 2010, Zha et al., 2013). Research aimed at improving adipose tissue function has focused on adipogenic transcription factors as key modifiable factors. PPAR- γ is one such transcription factor and has been shown to be significantly down regulated in response to ARVs, as well as in adipose tissue biopsies from HALS patients (Caron et al., 2009). Two PPAR- γ isoforms exist: PPAR- γ 1 which is found in almost all tissues except muscle and PPAR- γ 2 which is found primarily in adipose tissue and the intestine. The studies reported in this thesis refer to the PPAR- γ 2 isoform identified in adipose tissue.

1.2.3.1 Peroxisome proliferator-activated receptor gamma

PPAR- γ is a ligand-activated transcription factor and is expressed in abundance in adipose tissue (Desvergne and Wahli, 1999). PPAR- γ forms a heterodimer with retinoid-X-receptor (RXR) (PPAR:RXR) for DNA binding to the peroxisome proliferator response element (PPRE). The unbound PPAR-RXR is activated by a ligand and binds to the PPRE. The PPRE-bound heterodimer targets a coactivator-acetyltransferase complex to the promoter, which acetylates the histone tails and modifies promoter chromatin at the transcription initiation site region. Transcription factors are recruited to the accessible promoter and transcription is initiated (see **Figure 1.4**) (Desvergne & Wahli 1999).

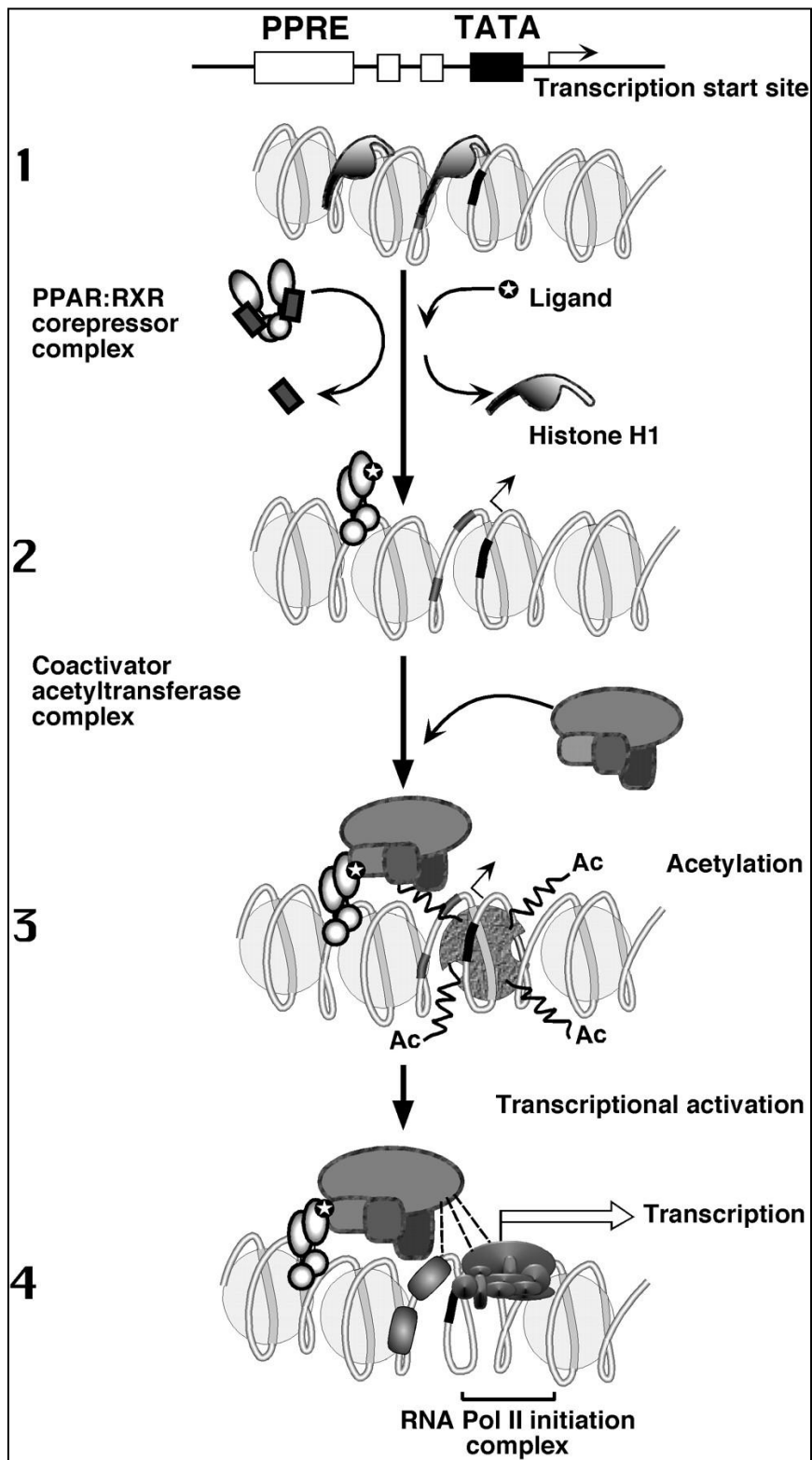


Figure 1.4 Model for transcriptional activation by PPARs
(Reproduced from (Desvergne and Wahli, 1999)).

1.2.3.1.1 PPAR- γ in adipogenesis

PPAR- γ has a number of important metabolic roles in adipose tissue (**Figure 1.5**) and is a major determinant of the adipocyte phenotype (Tontonoz et al., 1994). Through the process of adipogenesis, PPAR- γ activation induces differentiation of fibroblast-like pre-adipocytes into mature, lipid-enriched adipocytes (Otto and Lane, 2005). Activation of PPAR- γ induces the expression of another transcription factor CAAT-enhancer binding protein (CEBP)- α , and once expressed, CEBP- α and PPAR- γ positively regulate each other's expression during the adipogenic cascade via a positive feedback loop (Otto and Lane, 2005). These two transcription factors remain elevated throughout differentiation and the life of the mature adipocyte (Rosen, 2005). Other members of the CEBP family (CEBP- β and CEBP- δ) are expressed early in adipogenesis and induce the expression of CEBP α and PPAR- γ , both of which induce the terminal stages of differentiation.

During terminal differentiation, CEBP- α , PPAR- γ and sterol regulatory element binding protein (SREBP)-1c are involved in activating adipogenic genes responsible for maintaining the adipocyte phenotype (Otto and Lane, 2005). Adiponectin is one such target gene responsible for insulin signalling in adipocytes (Iwaki et al., 2003). Others include adipocyte lipid binding protein (aP2), fatty acid transport protein, phosphoenolpyruvate carboxykinase (PEPCK), as well as lipoprotein lipase (LPL) (Desvergne and Wahli, 1999). Lipid droplet-associated proteins, such as perilipin and cell death-inducing DFFA-like effector a (Cidea), were also found to have PPRE in their promoter regions and are involved in maintaining lipid droplet size once adipogenesis has proceeded (Targett-Adams et al., 2005, Viswakarma et al., 2007). PPAR- γ also appears to up-regulate the expression of glucose transporters, such as glucose transporter type 4 (GLUT4), which mediates insulin-stimulated glucose transport later in the differentiation process (Fernyhough et al., 2007).

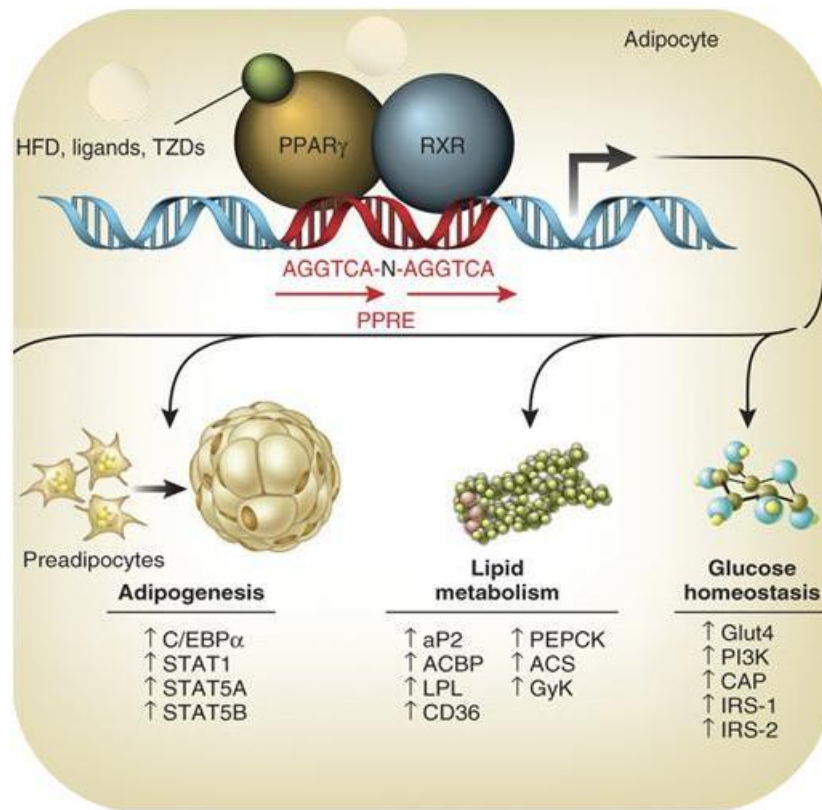


Figure 1.5 Multiple roles of PPAR- γ in adipose tissue

(Adapted from (Ahmadian *et al.*, 2013). HFD, high fat diet. TZD, thiazolidinedione. STAT, signal transducer and activator of transcription. ACBP, acyl-CoA-binding protein. CD36, cluster of differentiation 36. ACS, acyl-CoA-synthetase. GyK, glycerokinase. PI3K, phosphoinositide-3-kinase. CAP, c-Cbl associating protein. IRS, insulin receptor substrate.

1.2.3.1.2 Role of PPAR- γ in inflammation

PPAR- γ mediates its anti-inflammatory effects in a number of ways. PPAR- γ down-regulates the transcriptional activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) leading to repression of macrophage inflammatory genes (Monsalve et al., 2013). PPAR- γ ligands have been useful in helping to elucidate the mechanisms underlying the anti-inflammatory effects of the transcription factor. These include repression of pro-inflammatory cytokine and chemokine production from dendritic cells, repression of interferon gamma (IFN- γ) expression in activated T-cells and repression of chemokine expression in macrophages (**Figure 1.6**) (Straus and Glass, 2007). Recently, PPAR- γ was also found to regulate a novel population of regulatory T cells (Tregs) in VAT in mice (Cipolletta et al., 2012), which control VAT inflammation and IR (Feuerer et al., 2009).

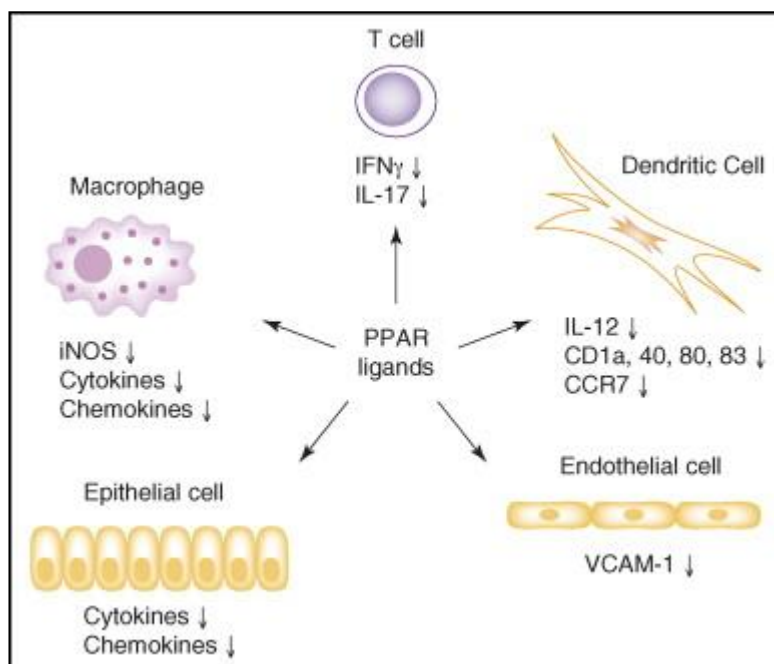


Figure 1.6 Anti-inflammatory actions of PPAR ligands
(Adapted from (Straus and Glass, 2007). CD1, cluster of differentiation 1a.
CCR7, C-C chemokine receptor 7. VCAM, vascular cell adhesion molecule.

1.2.3.1.3 Role of PPAR- γ in insulin sensitivity

During the early stages of adipogenesis, insulin triggers the induction of PPAR- γ . Once activated, PPAR- γ induces insulin-dependent GLUT4 expression, which transports glucose across the cell membrane, thereby regulating tissue glucose uptake (Wu et al 1998). Adipocyte dysfunction has been suggested to play a central role in the development of altered glucose homeostasis and IR; therefore, adipocyte dysfunction occurring as a result of down-regulated PPAR- γ is likely to contribute to IR (**Figure 1.7**). Support for this comes from *in vitro* studies of 3T3-L1 adipocytes, where PPAR- γ inhibition leads to reduced insulin-stimulated glucose uptake mediated by GLUT4 and GLUT1 (Liao et al., 2007), as well as reduced insulin receptor and insulin receptor substrate (Tamori et al., 2002).

In mouse models, fat-specific ablation of PPAR- γ results in increased blood glucose and serum insulin, as well as IR (Wang et al., 2013). Circulating free fatty acids (FFA) are associated with IR and PPAR- γ activation has been shown to lead to a reduction in FFA by increasing the number of small, insulin-sensitive adipocytes with greater lipid storage capacity (Saraf et al., 2012). The insulin sensitising effect of PPAR- γ may also be mediated by activation of target genes, such as adiponectin (Iwaki et al., 2003). PPAR- γ is a target for the family of anti-diabetic drugs thiazolidinediones (TZDs) (Lehmann et al., 1995). Pioglitazone, a member of the TZD family, was recently shown to decrease fasting plasma glucose and improve IR in patients with T2DM compared with placebo. This was accompanied by a significant increase in plasma adiponectin and a decrease in IL-6 suggesting that the insulin sensitising effect of this PPAR- γ ligand may be mediated by adipocytokines (Tripathy et al., 2013).

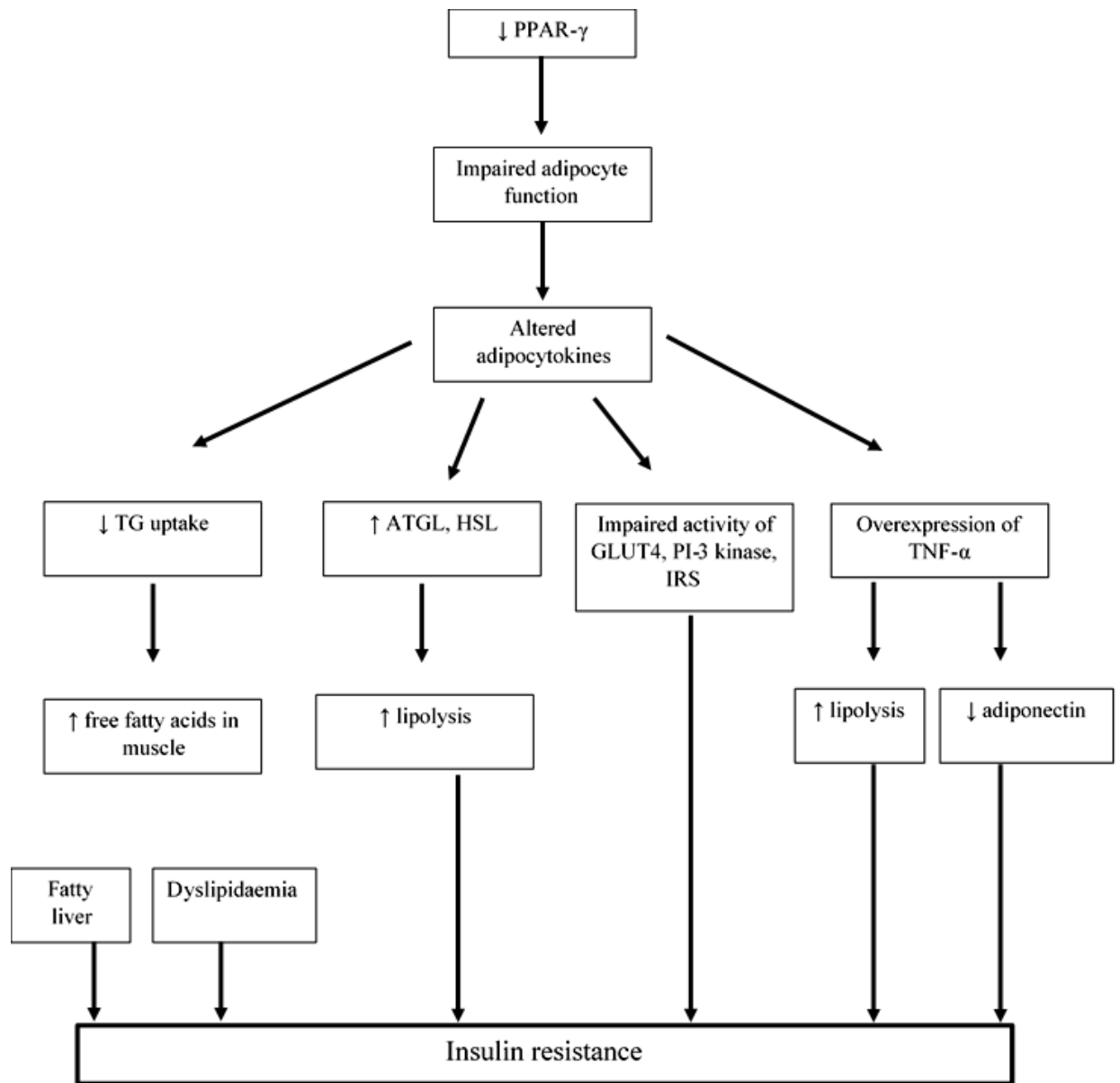


Figure 1.7 Putative role of PPAR- γ in insulin resistance (*Adapted from (Saraf et al., 2012)*). ATGL, adipose triglyceride lipase. HSL, hormone sensitive lipase. PI-3 kinase, phosphoinositide-3 kinase. IRS, insulin receptor substrate. TNF- α , tumour necrosis factor alpha.

1.2.3.1.4 PPAR- γ and HALS

As discussed previously, PPAR- γ is crucial in maintaining adipocyte function, glucose homeostasis and inflammatory profile. Mutations in the *PPARG* gene are associated with LD and symptoms of the MetS in humans (Ludtke et al., 2007). It is not surprising, therefore, that a role for PPAR- γ in the aetiology of HALS has been proposed (Caron et al., 2009). Down-regulation of PPAR- γ occurs as a result of HIV infection, as well as with the use of ARVs. HIV accessory protein, viral protein R, has been shown to suppress PPAR- γ activation *in vitro* in 3T3-L1 adipocytes by interacting with the ligand binding domain of the receptor (Shrivastav et al., 2008, Agarwal et al., 2013). HIV-1 Nef protein has also been shown to decrease the expression of PPAR- γ in human macrophages and T-cells (Otake 2004).

ARVs have been shown to decrease PPAR- γ expression and protein levels in some, but not all studies. A number of studies in mouse adipocytes, including a microarray analysis, have shown a significant reduction in PPAR- γ gene expression in response to PIs, NRTIs and NNRTIs (Caron et al., 2001, Caron et al., 2004, Pacenti et al., 2006, Viengchareun et al., 2007, Díaz-Delfín et al., 2011, Minami et al., 2011, Díaz-Delfín et al., 2012). In these studies, RTV was a particularly potent inhibitor of PPAR- γ at doses up to 20 μ M, but this finding is not confirmed by all studies (Grigem et al., 2005, Kim, 2006, Adler-Wailes et al., 2008). Similar findings for PPAR- γ down-regulation are observed for PIs and NRTIs in human pre-adipocytes derived from HIV-negative donors (Vernochet et al., 2005, Saillan-Barreau et al., 2008, Caso et al., 2010).

Adipose tissue biopsy samples from healthy, non-obese subjects show that PPAR- γ mRNA expression is significantly lower in VAT compared with SCAT (Lefebvre et al., 1998). In the context of HIV, patients with LA have lower mRNA concentrations of PPAR- γ and a 70% reduction in PPAR- γ protein concentration in SCAT compared with healthy controls (Bastard et al., 2002a). Similar findings have been observed in other studies and are accompanied by lower expression of PPAR- γ target gene adiponectin, as well as lower leptin, and increased IL-6 and tumour necrosis factor α (TNF- α) (Jan et al., 2004). PPAR- γ expression in SCAT was found to be significantly lower in HAART recipients compared with treatment-naïve individuals (Pace et al., 2003) and in patients with LD compared with those without LD (Kannisto et al., 2003). Interestingly, a six month interruption of PI therapy has been shown to significantly improve PPAR- γ

expression in SCAT samples from HIV-infected patients, suggesting that the effects on PPAR- γ may be reversible (Kim et al., 2007c).

The mechanisms underlying the reduction in PPAR- γ expression in response to ARVs reported in the majority of studies are not yet clear. The mitogen activated protein kinase/ extracellular signal-regulated protein kinase (MAPK/ERK) signalling pathway may play a role, as activation of the pathway enhances PPAR- γ expression (Prusty et al., 2002), and ARVs such as IDV and efavirenz have been shown to suppress ERK phosphorylation (Hong-Brown et al., 2004) and active ERK signalling (Dong et al., 2013).

1.2.3.2 Adiponectin

Adiponectin is a 30kDa protein secreted exclusively by adipocytes, which exists as three oligomeric isoforms (trimeric, hexameric and high molecular weight (HMW)) secreted into circulation as complexes (Scherer et al., 1995). The biological activity of adiponectin is mediated by the HMW form (Caselli et al., 2014). Adiponectin exerts its biological effects by binding to ubiquitously expressed receptors AdipoR1 and AdipoR2, as well as T-cadherin. Several endoplasmic reticulum molecules regulate the synthesis and secretion of adiponectin, and once in circulation, post-translational modifications maintain adiponectin stability (Caselli et al., 2014).

1.2.3.2.1 Regulation of adiponectin transcription

PPAR- γ positively regulates adiponectin gene expression through a PPRE in the adiponectin promoter (Iwaki et al., 2003). Synthetic PPAR- γ agonists, TZDs, have been shown to increase adiponectin transcription in 3T3-L1 adipocytes (Mineo et al., 2007) and in human subjects (Riera-Guardia and Rothenbacher, 2008). Weaker PPAR- γ agonists such as marine polyunsaturated fatty acids (PUFA) have also been shown to increase adiponectin mRNA and protein secretion in 3T3-L1 adipocytes (Oster et al., 2010). Similarly, conjugated linoleic acid (CLA) isomers have been shown to alter adiponectin expression via PPAR- γ -dependent mechanisms (Miller et al., 2008). Proinflammatory cytokines TNF- α and IL-6 negatively regulate adiponectin expression, which has been suggested to occur via a negative feedback loop (Mallewa, 2008).

1.2.3.2.2 Adiponectin, insulin sensitivity and HALS

Adiponectin is secreted by adipocytes in response to insulin via a phosphoinositide 3-kinase (PI3K)-dependent mechanism (Blumer et al., 2008). In humans, raised adiponectin has been shown to increase insulin sensitivity (Gao et al., 2013). Adiponectin exerts its insulin sensitising effects by increasing glucose uptake by skeletal muscles (Liu and Sweeney, 2014), inhibiting gluconeogenic enzymes and glucose production in the liver and increasing insulin gene expression and secretion in pancreatic beta cells (Tao et al., 2014). Adiponectin also works indirectly to decrease plasma FFA and reduce ectopic lipid accumulation by stimulating acetyl-CoA carboxylase and fatty acid β -oxidation (Yamauchi et al., 2002). Recently, a novel adapter protein APPL1 has been identified as a key mediator of the insulin sensitising effects of adiponectin in these tissues (Cheng et al., 2014). Proinflammatory cytokines such as TNF- α suppress adiponectin transcription and induce IR by promoting the expression of insulin-like growth factor binding protein-3 (Kim et al., 2007b).

Adiponectin has been investigated extensively in HALS, where hypoadiponectinaemia is a common finding (Mynarcik et al., 2002, Kosmiski et al., 2003a, Bezante et al., 2009, Luo et al., 2009, Veloso et al., 2012). Plasma adiponectin levels in patients with LD are significantly lower compared with individuals without LD and healthy controls (1.6 vs. 3.4 $\mu\text{g/ml}$ and 1.6 vs. 6.7 $\mu\text{g/ml}$, respectively) (Kosmiski et al., 2003a). Adiponectin concentration has been observed to change over time in individuals with LD, increasing up until 6 months after initiation of HAART and then decreasing substantially over time until month 30, when the reduction is maintained (Luo et al., 2009).

Adiponectin concentration has been shown to positively correlate with measures of fat distribution (Kosmiski et al., 2003a), insulin sensitivity (Mynarcik et al., 2002) and negatively correlated with IR, BMI, WHR, TG and high sensitivity-CRP (Vigouroux et al., 2003). Use of older ARVs is associated with hypoadiponectinaemia (Lindegaard et al., 2004). Adipose tissue biopsy samples from patients with peripheral LA show a 2-fold lower adiponectin mRNA expression compared with healthy individuals (Jan et al., 2004). Another study demonstrated increased adiponectin expression in adipose tissue from patients switching from older (d4T) to newer (raltegravir) ARVs (Domingo 2014). *In vitro*, treatment with ARVs has shown inconsistent results: RTV in particular has been shown to reduce adiponectin mRNA expression and secretion by up to 50% in

3T3-L1 and Simpson Golabi Behmel Syndrome (SGBS) adipocytes (Grigem et al., 2005, Kim, 2006, Pacenti et al., 2006, Lagathu, 2007, Adler-Wailes et al., 2008, Díaz-Delfín et al., 2011), and has been suggested to occur as a result of increased oxidative stress (Lagathu, 2007) and negative feedback by inflammatory cytokines (Luo et al., 2009). However, in other studies ARVs increase adiponectin expression (Díaz-Delfín et al., 2011), or have no effect (Jones et al., 2008).

1.2.3.3 Perilipin

Perilipin-1 (Plin1) is expressed in white adipose tissue (WAT) and is one of five members of the perilipin, adipophilin and TIP47 family of proteins, responsible for protecting the lipid droplet from lipolysis. Plin1 covers the surface of the lipid droplet and has recently been suggested to be involved in increasing lipid droplet size (Sztalryd and Kimmel, 2014). Plin1 expression is regulated by the PPAR:RXR heterodimer via a PPRE in the Plin1 promoter (Dalen et al., 2004). PPAR- γ agonists have differential effects on Plin1 expression; synthetic PPAR- γ agonist rosiglitazone increased Plin1 expression in rat SCAT (Kim et al., 2007a), while natural putative PPAR- γ agonist, docosahexanoic acid (DHA), reduced Plin1 expression by 49% in 3T3-L1 adipocytes compared with control (Barber et al., 2013). In a recent randomised controlled trial, a diet high in saturated fat and a diet supplemented with omega-3 PUFA (n-3 PUFA) were both shown to decrease Plin1 expression in SCAT samples from MetS patients (Camargo et al., 2014). The authors suggest a reduction in PPAR- γ may be responsible for this effect. The beneficial role of Plin1 has been demonstrated in animal models, where over-expression of adipose Plin1 in mice fed a high fat diet results in resistance to obesity and improved insulin sensitivity (Miyoshi et al., 2010).

In the context of HALS, Plin1 expression is negatively altered by ARVs. PI treatment decreased Plin1 expression in human SCAT, but not VAT (Leroyer et al., 2011). A similar study in rats treated with PIs showed decreased Plin1 expression in fat isolated from SC fat pads (56.8%) and perigonadal fat pads (20.5%) (Kovsan et al., 2008). Similar findings are observed *in vitro*, with 3T3-L1 adipocytes and preadipocyte cell lines from rats showing decreased Plin1 expression in response to PIs (Rudich et al., 2001, Adler-Wailes et al., 2005, Kovsan et al., 2008). The effects of ARV on depot specific expression of Plin1 may be one mechanism underlying LA observed in patients with HALS.

1.2.3.4 Cell-death inducing DFFA-like effector a

Cidea is another lipid droplet-associated protein expressed mainly in adipocytes, which promotes lipid storage in large lipid droplets (Xu et al., 2012). Cidea is thought to facilitate enlargement of lipid droplets by destabilising phospholipids at contact sites between lipid droplets, thereby allowing lipid exchange and transfer (Gong et al., 2011). Mice deficient in Cidea have a lean phenotype with significantly reduced adipose tissue (Wu et al., 2014). The Cidea gene promoter contains a PPRE and transcription has been shown to be regulated by PPAR- γ (Puri et al., 2008). Interestingly, natural putative PPAR- γ agonists, eicosapentanoic acid (EPA) and DHA have been shown to reduce Cidea expression in 3T3-L1 adipocytes (Barber et al., 2013). In the context of HIV, Cidea has been shown to be decreased in microarray analysis of adipocytes treated with 10 μ M RTV (Adler-Wailes et al., 2008). One study has investigated the expression of Cidec, another member of the Cide family, in human preadipocyte cells treated with the PI, saquinavir, and found it to be significantly decreased (Bociaga-Jasik et al., 2013).

1.2.3.5 Leptin

Leptin was first discovered in 1994 as a product of the mouse Ob gene (Zhang et al., 1994) and since then has been a target in efforts to tackle obesity. Leptin is a 16 kDa polypeptide secreted by WAT, which circulates at concentrations reflective of SCAT stores (Pan et al., 2014). Although obesity and IR are associated with hypoleptinaemia, increased leptin levels have been identified in some obese patients, which has been suggested to occur as a result of leptin resistance (Post et al., 2014). The role of leptin in counteracting obesity is mediated via leptin receptors located in the brain, which regulate feeding behaviour and energy expenditure (Pan et al., 2014). Leptin also modulates adipocyte metabolism directly via leptin receptors located on the adipocyte, as well as indirectly via the action of insulin on adipocytes (Harris, 2014).

Leptin increases in proportion to decreasing adipose tissue stores and is therefore of interest in HALS, where abnormalities of body fat distribution are characteristic features. *In vitro*, ARVs have been shown to significantly reduce leptin expression in human adipocytes (Díaz-Delfín et al., 2011, Díaz-Delfín et al., 2012). RTV, a PI, also decreases leptin levels in mice (Vyas et al., 2010). In contrast, treatment of SC adipocytes from HIV-negative men with RTV increased leptin secretion (Jones et al., 2008). Leptin levels have been shown to be significantly reduced in patients with HALS compared with those without HALS and uninfected controls (3.5 vs. 4.6 vs. 8.2 ng/ml,

respectively) (Veloso et al., 2012). However, other groups have shown raised leptin levels to be associated with increased WC (de Luis et al., 2012) and IR (Arama et al., 2013) and a three-fold increased risk of peripheral LA, but not abdominal LH (Calmy et al., 2008). Conflicting findings may reflect the fact that leptin levels correlate with body fat stores, which are altered in different adipose tissue depots in HALS.

Leptin administration has been investigated in an effort to correct hypoleptinaemia in HALS. Leptin administration in mice was found to reverse RTV-induced elevations in plasma TC, while in the same study a PUFA-rich diet had no effect (Riddle et al., 2003). In patients with HALS, recombinant human leptin administration decreased VAT by 32% and TG, TC and LDL-C by 39%, 15% and 20%, respectively, after 6 months (Mulligan et al., 2009). Similar findings for leptin administration and TC have been demonstrated by others in hypoleptinaemic, hypertriglyceridaemic patients with HALS (Sekhar et al., 2012). Although results are somewhat inconsistent, evidence exists to support a role for leptin in the pathogenesis of body fat abnormalities in HALS.

1.2.3.6 Resistin

Resistin is a 12.5 kDa cysteine-rich protein secreted in rodents by adipocytes and in humans by macrophages (Steppan et al., 2001, Park and Ahima, 2013). Resistin expression in mice is regulated by PPAR- γ , which is illustrated by the suppression of resistin gene expression in 3T3-L1 adipocytes by PPAR- γ agonists, TZDs (Chung et al., 2006). Resistin was originally postulated to be the link between obesity and IR, and studies have shown improved insulin sensitivity and glucose levels associated with loss of resistin in obesity. Furthermore, resistin is secreted by adipocytes and macrophages in response to chronic inflammation, a characteristic feature of obesity and T2DM (Stofkova, 2010). Serum resistin has been shown to correlate with degree of VAT and was shown by some (Degawa-Yamauchi et al., 2003), but not all (Lee et al., 2003) studies to be higher in obese individuals.

In the context of HIV, plasma resistin levels have been shown to be significantly greater in HIV-infected individuals without LD compared with those with LD and uninfected individuals (4.21 vs. 3.64 vs. 2.74 ng/ml, respectively), and in patients with isolated LA compared with those with a mixed form of LD (4.64 vs. 3.27 ng/ml) (Escote et al., 2011). These findings are supported by another group (Arama et al., 2014). PPAR- γ agonist rosiglitazone was previously shown to decrease serum resistin (12.17 to 10.23

ng/ml) in HIV-infected individuals with LA and hyperinsulinaemia (Kamin et al., 2005). Furthermore, a SNP in the resistin gene was recently found to increase the risk of metabolic complications associated with ARVs (Ranade et al., 2008). Although these studies demonstrate a role for resistin in HALS, others have found no association between serum resistin and any form of LD or metabolic abnormality in HIV-infected individuals receiving HAART (Barb et al., 2005). It is clear that resistin is involved in HALS, but the degree of involvement and its specific role remains to be elucidated.

1.2.3.7 Interleukin-6

IL-6 is a single 21-28 kDa polypeptide chain found to have both pro- and anti-inflammatory properties. In humans, 15-35% of circulating IL-6 derives from adipose tissue and is primarily secreted by non-adipose cells such as macrophages (Eder et al., 2009). IL-6 transcription is regulated by NF- κ B and CEBP- α , and is also subject to post-translational modifications (Rincon, 2012). Additionally, TNF- α and adiponectin have been shown to regulate IL-6 production in adipocytes, via NF- κ B, PPAR- γ and CEBP- β mediated mechanisms (Ito et al., 2009, Zoico et al., 2009).

The role of IL-6 in IR is unclear. IL-6 has been shown to inhibit the action of insulin in adipocytes and mice, and IL-6 knockout mice have disrupted glucose metabolism. Furthermore, IL-6 secretion in skeletal muscle is increased following exercise, which is associated with improved insulin sensitivity (Pal et al., 2014). IL-6 is increased in obese individuals compared with lean controls and decreases in response to weight loss (Bastard et al., 2000). Recently, it has been shown that increased IL-6 in VAT enhances diet-induced obesity and inflammation by suppressing Tregs, a novel population of T cells, which regulate inflammation and IR (Priceman et al., 2013).

The importance of IL-6 in HIV is demonstrated by the observation that IL-6 levels at the time of seroconversion predict HIV disease progression (Hamlyn et al., 2014). HIV-infected patients have higher plasma IL-6 compared with uninfected controls (2.6 vs. 1.4 pg/ml) and a higher prevalence of SNPs in the IL-6 gene (Saumoy et al., 2008). Patients with LD were found to have higher plasma IL-6 compared with those without LD (1.5 vs. 1.13 pg/ml) (Crawford et al., 2013). The role of HAART in mediating increases in IL-6 was demonstrated by Kim *et al* (2007c) who showed that interruption of HAART for 6 months resulted in fewer IL-6 expressing cells in adipose tissue. *In vitro*, treatment with PIs (IDV, nelfinavir, lopinavir, RTV) and NRTI (d4T and ZDV)

increases IL-6 expression in human adipocytes (Lagathu, 2007), a finding corroborated by others in both human and murine adipocytes (Grigem et al., 2005, Jones et al., 2005, Lagathu et al., 2005, Kim, 2006).

In vivo, SCAT from HALS patients shows a three-fold increase in IL-6 mRNA compared with healthy controls (Lihn et al., 2003). This finding is supported by other groups (Kannisto et al., 2003, Johnson et al., 2004, Saumoy et al., 2008, Gallego-Escuredo et al., 2013) and was found to be associated with apoptosis (Jan et al., 2004). Interestingly, a recent study found reduced IL-6 protein expression in VAT from patients with LD compared with uninfected controls (Gallego-Escuredo et al., 2013). Regardless of the presence or absence of LD, serum IL-6 was found to be positively associated with VAT and negatively associated with lower limb SCAT (Johnson et al., 2004). IL-6 has also been shown to correlate positively with WHR in patients with HIV (Vigouroux et al., 2003). Treatment with PPAR- γ agonist rosiglitazone for 24 weeks resulted in a three-fold reduction in IL-6 expression in SCAT from HALS patients (Sutinen et al., 2004), highlighting the role of PPAR- γ in IL-6 regulation. In summary, IL-6 expression is altered in HALS which may be due to a combination of host genetic factors, HIV infection itself and HAART. Increased circulating levels may contribute to inflammation in HALS.

1.2.3.8 Mitochondrial toxicity

Mitochondrial toxicity is another mechanism involved in the pathogenesis of LA in HIV. Unlike classical mitochondrial disease, which is caused by a mutation in nuclear or mitochondrial DNA, mitochondrial toxicity in HIV is caused by NRTI-induced inhibition of mitochondrial DNA polymerase γ , a key enzyme responsible for DNA replication. NRTIs have a high affinity for mitochondrial polymerase γ and are incorporated during mitochondrial DNA replication resulting in chain termination of mitochondrial polymerase γ (Gardner et al., 2013). HIV RT has also been shown to have a high affinity for mitochondrial polymerase γ . Human studies in support of this show an association between HIV infection and increased mitochondrial toxicity independent of ART and LD (Garrahou et al., 2011), but an association has also been found for ART (McComsey et al., 2008). PIs and NNRTIs also cause mitochondrial toxicity without affecting polymerase γ , which has been recently suggested to occur via indirect competition with endogenous deoxynucleotide triphosphates (Selvaraj et al., 2014).

1.2.4 Management of HALS

1.2.4.1 Medication and surgery

Current pharmacological management of HALS focuses on the correction of dyslipidaemia, T2DM, HTN and abdominal obesity. Guidelines from the European AIDS Clinical Society (EACS) for the management of dyslipidaemia in HIV (Lundgren et al., 2008) are summarised in **Table 1.5**. If initial management with diet and lifestyle proves ineffective, switching ART is recommended, followed by use of statins. Switching to more “lipid-friendly” ART is often used to manage dyslipidaemia. Common switch strategies include switching to atazanavir from another PI, which has been shown to improve TG and TC, switching from thymidine analogues to TDF-based regimens within the NRTI family and switching from PI to NNRTI (Lake and Currier, 2010).

Table 1.5 Management of dyslipidaemia in HIV

Lipid abnormality	First treatment option	Additional therapy
LDL-C > 5 mmol/L	Statin	+ Ezetimibe
LDL-C > 5 mmol/L & TG 5-10 mmol/L	Statin	+ fibrate (/nicotinic acid derivative)
TG 2.3-10 mmol/L	Diet; no alcohol	-
TG > 10 mmol/L	Fibrate	+ omega 3 acid ester (or nicotinic acid derivative)
HDL-C < 0.9 mmol/L	Fibrate	+ nicotinic acid derivative

Adapted from (Lundgren et al., 2008). LDL-C, low-density lipoprotein cholesterol. TG, triglyceride. HDL-C, high-density lipoprotein cholesterol.

Pharmacological interventions to control glucose in T2DM include metformin, TZDs and insulin. TZDs are potent PPAR- γ agonists and have been shown to improve metabolic and body composition abnormalities in HALS including ameliorating IR, increasing HDL-C, decreasing TG (Walli et al., 2000, Slama et al., 2008), increasing SCAT and decreasing VAT (Walli et al., 2000, Raboud et al., 2010). However, some studies demonstrated negative effects of TZDs on TC and LDL-C (Walli et al., 2000), as well as no effect on LA (Carr et al., 2004). Other drugs acting via a PPAR- γ dependent mechanism include the anti-inflammatory drug leflunomide, which was

found to significantly improve LA and dyslipidaemia associated with RTV (Mencarelli et al., 2012).

Tesamorelin (Egrifta[™]) is a growth hormone releasing factor and is the only drug approved for the treatment of abdominal obesity associated with ART. However, its use is associated with side-effects, which include worsening glycaemic control, and so the benefits should be weighed against the risks of growth hormone treatment (Sivakumar et al., 2011).

Surgery is frequently used to correct body fat abnormalities associated with HALS. Facial LA is probably the most stigmatising feature of HALS and its correction typically involves the use of biodegradable or non-biodegradable fillers, or fat transplantation (Guaraldi et al., 2011). LH of the neck and trunk can be corrected using ultrasonic liposuction and excisional lipectomy (Hultman et al., 2007). As with all surgical interventions, there is a risk of post-operative complications.

1.2.4.2 Exercise

Exercise also forms part of the EACS guideline for the management of metabolic diseases in HIV and has recently been shown to be an independent protective factor against LD (Della Justina et al., 2014). A recent systematic review of randomised controlled trials found that resistance exercise increased LBM and reduced body weight, while aerobic exercise reduced body weight, body fat and WHR in adults with HIV (Gomes-Neto et al., 2013). These findings are confirmed by an earlier systematic review and meta-analysis which also found decreased WC in response to aerobic exercise compared with control (-2.60 cm vs. +0.03 cm) (Fillipas, 2010). Furthermore, trunk fat was found to decrease significantly after 6 weeks of moderate-intensity exercise in HIV-infected men with greater than 20% body fat compared with non-intervention controls (33.7% vs. 32.2%) (Dudgeon et al., 2012). In terms of metabolic abnormalities, TC, TG, FG and insulin were all found to be reduced in response to exercise (Fillipas, 2010). Overall, it appears that exercise may be beneficial in improving lipid and glucose parameters, as well as central adiposity in HIV-infected individuals.

1.2.4.3 Diet

Nutrition plays an important role in maintaining health in HIV-infected individuals. However, according to a consensus statement from the American Dietetic Association, evidence for the role of diet in mitigating HALS is limited (American Dietetic Association, 2010). The effect of diet in mitigating the metabolic and morphological abnormalities of HALS has been investigated in a number of intervention studies (**Table 1.6**).

An early case report showed reduced abdominal fat and improved metabolic profile in one male with LD after 4 months of a low-fat, low-glycaemic index, high fibre diet plus exercise (Roubenoff et al., 2002). Another group found that a low fat diet for 6 months was beneficial in reducing TC by 10% and TG by 23% among HIV-infected individuals with hyperlipidaemia (Barrios et al., 2002). In contrast, another study found increased TG (+1.3 mmol/L) and no effect on TC following a low-fat diet for 1 year (Ng et al., 2011). Another study showed that a low fat diet in combination with aerobic exercise for 12 weeks significantly reduced body weight (-2 kg), body fat percentage (-5%) and WHR in hyperlipidaemic HALS patients (Terry et al., 2006). A 24-week trial of low fat diet combined with exercise and niacin (up to 2 g/d) caused a significant increase in plasma HDL-C (+0.11 mmol/l) and reduced TC:HDL-C ratio (4.9 vs. 4.3) in patients treated with HAART (Balasubramanyam et al., 2011). A 12 month intervention with the NCEP diet (25-35% total energy from fat, < 7% from saturated fat, 50-60% of energy from carbohydrate, 15% from protein and 20-30 g/d fibre) decreased plasma TG (-0.38 mmol/l) and reduced the proportion of patients with a dyslipidaemic lipid profile compared with control (21% vs. 68%) (Lazzaretti et al., 2012).

In terms of a Mediterranean-style diet, adherence to the diet, as defined by a high Mediterranean Diet Score, was found to have no effect on serum lipids in one study (Turčinov et al., 2009) and was positively associated with HDL-C and marginally negatively associated with TG in another study (Tsiodras et al., 2009). Moreover, a pilot randomised controlled trial (RCT) investigating the effects of a Mediterranean diet in LD patients showed increased TC (+0.52 mmol/l) after 12 months (Ng et al., 2011). Overall, existing evidence appears to indicate the potential benefit of a diet high in fibre, low in saturated fat, which includes polyunsaturated fat, and which corresponds with a Mediterranean style dietary pattern in mitigating aspects of HALS.

Table 1.6 Dietary interventions to improve metabolic abnormalities in HIV

Reference	Design	Type of intervention	Subject characteristics	Duration	Outcome
Diet					
Barrios <i>et al</i> (2002)	PIS	Low fat diet	230 HIV+, receiving ART, with dyslipidaemia.	6 m	↓TC, TG & weight
Lazzeretti <i>et al</i> (2012)	RCT	NCEP diet (low-fat, high fibre)	43 randomised to ART + diet; 40 randomised to ART only	12 m	↓ TG (25%); ↓ dyslipidaemia vs. control (21 vs. 68%)
Ng <i>et al</i> (2011)	Pilot RCT	Modified Mediterranean diet vs. low-fat, low-cholesterol diet	48 HIV+	12 m	Mediterranean: no Δ TG, ↑ TC. Low fat: no Δ TC, ↑ TG
Roubenoff <i>et al</i> (2002)	CR	Moderate fat, low GI, high fibre diet + exercise three times per week.	1 HIV+, M, receiving ART.	4 m	↓ total & trunk fat, LDL-C, TC, glucose & IR.
Terry <i>et al</i> (2006)	RCT	Low fat diet + aerobic exercise/ low fat diet only	30 HIV+, 20M, HALS, receiving ART.	12 w	↓ body weight, body fat & WHR in two groups
Supplements					
Balasubramanyam <i>et al</i> (2011)	RDPCT	Diet & exercise + niacin (2g/d)	191 HIV+, ↑TG	24 w	↑ HDL-C & adiponectin
Benedini <i>et al</i> (2009)	RIT	2 g/d L-acetylcarnitine	9 HIV+/9 healthy controls, receiving PI/NRTI	8 m	↑ % leg fat
Calmy <i>et al</i> (2010)	PRT	36 g uridine t.d.s	HIV+, M, LA+	60 d	No Δ limb fat mass
Marcel <i>et al</i> (2011)	RPS	19 g/d Spirulina (algae) vs. soybean	33 HIV+, insulin resistant	8 w	Improved insulin sensitivity
Metkus <i>et al</i> (2013)	RDPCT	3.6 g/d n-3 PUFA or corn oil	HIV+, hyperTG	8 w	↓TG and IL-6

Table 1.6 continued

Reference	Design	Intervention/ placebo	Subjects/ controls	Duration	Outcome
McComsey <i>et al</i> (2007)	POL	36 g uridine t.d.s. every other day	14 HIV+, 11M, receiving ART, LA+.	16w + 16 w washout	LA scores by patient and physician improved
Oliveira <i>et al</i> (2013)	RPCT	3 g/d n-3 PUFA or soy oil	83 HIV+, 64M, receiving ART	24 w	No effect on lipids or WC
Sutinen <i>et al</i> (2007)	RDPCT	36 g uridine t.d.s.	20 HIV+, 17M, 10 cases, 10 controls, receiving ART, LA+	3 m	↑total limb fat, intra-abdominal fat & total body fat

Δ, change. PIS, prospective intervention study. ART, antiretroviral therapy, m, months. TC, total cholesterol. TG, triglyceride. RCT, controlled trial. NCEP, National Cholesterol Education Programme. CR, case report. GI, glycaemic index. M, male. LDL-C, low-density lipoprotein cholesterol. IR, insulin resistance. RCT, randomised controlled trial. HALS, HIV-associated lipodystrophy syndrome. w, weeks. WHR, waist:hip ratio. RDPCT, randomised, double blinded, placebo controlled trial. HDL-C, high-density lipoprotein cholesterol. RIT, randomised intervention trial. PI, protease inhibitors. NRTI, nucleoside reverse transcriptase inhibitors. PRT, prospective, randomised trial. t.d.s., ter die sumendum (three times a day). LA+, lipoatrophy. d, days. POL, prospective open label. RPCT, randomised placebo controlled trial.

Nutritional supplementation has also been investigated in HALS (**Table 1.6**). Supplementation with uridine has been shown to increase limb fat by 7% (18% to 25%) after 3 months. However, intra-abdominal and total fat were also significantly increased in the same study compared with placebo (+210 g vs. -80 g and +1920 g vs. 240 g, respectively) (Sutinen et al., 2007). Increases in lean body mass were demonstrated after 24 weeks of the same supplement (Calmy et al., 2010). Findings from a recent systematic review show that nicotinic acid analogues significantly reduce TG (-0.54 vs. +0.65 mmol/l) and increase HDL-C (+0.08 vs. -0.03 mmol/l) compared with placebo, while chromium supplementation significantly improves IR and reduces TG (-0.78 vs. +0.14 mmol/l) in patients with LD compared with those without LD (Stradling et al., 2012). Supplementation with 19 g/d Spirulina, a blue-green algae, significantly improved insulin sensitivity after 8 weeks compared with soybean in HIV-infected individuals (Marcel et al., 2011).

N-3 PUFA, which are natural putative PPAR- γ agonists, have been investigated for their role in the prevention of dyslipidaemia in HIV. A recent systematic review and meta-analysis of seven studies investigating the effect of n-3 PUFA supplementation (0.3 g/d to 4.86 g/d for 12-24 weeks) on dyslipidaemia in HIV showed an overall significant reduction in fasting TG (-1.12 mmol/l) in the intervention group compared with control. N-3 PUFA supplementation also significantly reduced TC (-0.36 mmol/l), but had no effect on HDL-C and LDL-C (Stradling et al., 2012). More recently, a study of hypertriglyceridaemic HIV patients showed that 3.6 g/d of n-3 PUFA for 8 weeks significantly decreased TG (-0.38 vs. +0.45 mmol/l) and caused a 39% reduction in IL-6 (-0.62 vs. +0.18 pg/ml) compared with a corn oil placebo (Metkus et al., 2013). In contrast, another study compared 3 g/d n-3 PUFA with 3 g/d soy oil for 24 weeks and found no significant effect on lipid parameters or WC (Oliveira et al., 2013). Although heterogeneity exists between the studies in terms of the study population, supplements used and duration, it appears that n-3 PUFA supplementation is beneficial in mitigating dyslipidaemia in HIV.

1.3 Conjugated linoleic acid

1.3.1 Biosynthesis

CLA is the term used to describe a group of at least 28 positional and stereo-isomers of linoleic acid with 18 carbons and two unsaturated double bonds separated by one single bond (Lawson et al., 2001). CLA isomers are formed by incomplete biohydrogenation of linoleic acid by the microorganism *Butyrivibrio fibrisolvens* in the rumen (Bauman et al., 1999). CLA can also be formed in bovine mammary tissue by the action of delta 9 – desaturase on vaccenic acid (Griinari et al., 2000). The isomers differ in the location of their double bonds and position of the hydrogen atom. The hydrogen atom at each double bond can be positioned in the *cis* or *trans* configuration.

The main naturally occurring isomer consists of two unsaturated double bonds located at positions 9 and 11 from the carboxylic end. CLA concentration of milk and animal fat can be enhanced by modifying the CLA content of animal feed to include marine algae or plant oils, adjusting the proportion of grazed grass in the diet and lowering forage: concentrate ratios (Lawson et al., 2001). Animal genetics may also play a role in determining CLA content of milk and animal fat. Type of feed influences the extent of biohydrogenation in the rumen and therefore, the concentration of CLA (Lawson et al., 2001). Grass fed animals have up to a 5-fold increased *cis*-9,*trans*-11 (c9,t11) concentration in their milk compared to animals fed a usual diet consisting of a 50:50 mix of forage and grain (Dhiman et al., 2005). Supplementation of the animal's diet with plant oils high in linoleic acid (sunflower, soya bean and rapeseed) may also increase milk CLA composition (Lawson et al., 2001).

1.3.2 Industrial synthesis

Synthetic CLA supplements are used extensively in human studies and typically contain a mix of the two main isomers c9,t11 and *trans*-10,*cis*-12 (t10,c12) in a 1:1 ratio. The final concentration of CLA depends on the initial level of linoleic acid (Kapoor et al., 2005). Synthetic CLA is either directly derived from safflower oil or undergoes enzymatic pre-processing to increase the content of linoleic acid. Alkaline treatment of the oil results in saponification and isomerisation (conjugation) leading to the formation of c9,t11 and t10,c12 in a 1:1 ratio. Subsequently, dilution and acidification of the

mixture, followed by washing, drying and distillation, results in the formation of FFA, which are finally re-esterified with glycerol (Kapoor et al., 2005, EFSA, 2010).

1.3.3 Dietary sources

The c9,t11 and t10,c12 isomers appear to be the most biologically active CLA isomers (Pariza et al., 2001). The c9,t11 isomer is more predominant in the diet, constituting 90% of total CLA and is found mainly in meat and dairy products derived from ruminants (Mushtaq et al., 2010). The average intake of c9,t11 from dietary sources is estimated at 97.5 mg/day in the UK (Mushtaq et al., 2010) and 151-212 mg/d in the US (Ritzenthaler et al., 2001). The c9, t11 content of some of these food items can be found in **Table 1.7**.

Table 1.7 Main dietary sources of c9,t11-CLA (mg/100 g food)

Milk and milk products	
Milk-full fat	7.1
Milk- semi-skimmed	3.3
Milk-skimmed	-
Double cream	176.2
Crème fraiche	162.9
Meat and meat products	
Minced lamb	276.8
Beef rib eye steak	178.6
Beef mince	51.2
Butter and margarine	
Butter	201.0
Olive oil	4.7
Polyunsaturated margarine	3.5
Cheese	
Medium Irish cheddar	443.8
Parmesan	226.4
Stilton	192.5
Feta	110.5
English Brie	94.9
Goat's cheese	71.3

Adapted from (Mushtaq et al., 2010)

1.3.4 Biological activities

The biological activities of CLA include modulation of immune and inflammatory responses, CVD protection, cancer prevention, bone health, and anti-obesity effects, which will be discussed below in greater detail.

1.3.4.1 Immune and inflammatory responses

The immunomodulatory and anti-inflammatory effects of CLA have been investigated in *in vitro*, animal and human studies. In macrophages, c9,t11 has been shown to exert anti-inflammatory effects by significantly suppressing membrane and lipid raft CD14 and LPS-induced interferon regulatory factor-3 activation, as well as decreasing LPS stimulated endocytosis of toll like receptor 4, a protein involved in antigen detection and immune system activation (Dowling et al., 2013). Another study demonstrated a decrease in cell adhesion molecules in human vein endothelial cells treated with c9,t11 and t10,c12 (Stachowska et al., 2012). The authors of this study suggest that this may be one mechanism underlying the anti-atherosclerotic effects of CLA. Human bronchial cells and eosinophils play an important role in inflammatory airways disease. CD69 and CD13 are induced by the activation of T lymphocytes and NK cells and are responsible for lymphocyte proliferation and function. c9,t11 has been shown to significantly reduce the activation markers in human bronchial cells and eosinophils. The effects of c9,t11 in this study were mediated through activation of PPAR- γ (Jaudszus et al., 2005). A study in mice showed that intestinal probiotic bacteria produced CLA, which decreased myeloid cell PPAR- γ leading to suppression of colitis (Bassaganya-Riera et al., 2012).

A dietary intervention study investigating the effect of pecorino cheese for 10 weeks in healthy subjects found a reduction in inflammatory cytokines IL-6, IL-8 and TNF- α (Sofi et al., 2010). In overweight, mild asthmatics, 12 weeks of CLA supplementation (4.5 g/d mixed isomers) in addition to usual treatment resulted in a significant improvement in airway responsiveness (MacRedmond et al., 2010). In another study, twelve weeks of c9,t11 supplementation (2 g/d; 65% c9,t11 and 8.5% t10,12) in subjects with birch pollen allergy led to a decrease in the production of TNF- α and IFN- γ (Turpeinen et al., 2008). A study in healthy men showed that 2.2 g/d of an isomeric mixture of CLA isomers for 8 weeks reduced *ex vivo* peripheral blood mononuclear cells IL-2 secretion, a cytokine produced by T cells during an immune challenge (Mullen et al., 2007). CLA supplements have also been tried in certain disease states. In patients with Crohn's disease, 6 g/d CLA reduced pro-inflammatory cytokine

production from CD4+ and CD8+ cells, and decreased disease activity (Bassaganya-Riera et al., 2012). In patients with active rheumatoid arthritis, CLA was shown to improve the clinical manifestations of the disease after 3 months of supplementation with 2 g/day of mixed CLA isomers (Aryaeian et al., 2009). Although these studies have demonstrated a beneficial effect of CLA, some have failed to find any effect (Smit et al., 2011), or have demonstrated a negative effect, particularly on levels of C-reactive protein (Gaullier et al., 2007). To the best of our knowledge, no studies have investigated the effects of CLA on markers of immune function in HIV-infected individuals.

1.3.4.2 Cardiovascular disease

Unlike non-conjugated trans fatty acids found in snacks and fast food, ruminant trans fatty acids have shown beneficial effects in CVD (Bendsen et al., 2011). Evidence from animal studies has shown CLA to be beneficial in reducing atherosclerosis (Toomey et al., 2006, Mitchell et al., 2012), TG and TC (Hur et al., 2013), LDL-C and increasing HDL-C (Noto et al., 2006). However, results are conflicting, as some have shown CLA to increase TG and LDL-C (Verma and Kansal, 2013) and promote atherosclerotic lesion development (Reynolds et al., 2013). A number of studies in humans have investigated the effect of CLA mixed isomer supplementation on blood lipids (**Table 1.8**). HDL-C was reduced in healthy overweight subjects (Gaullier et al., 2004) and obese men with MetS (Risérus et al., 2002), but increased in type 2 diabetics (Moloney et al., 2004) in response to mixed CLA isomer supplementation. Another study demonstrated increased TC:HDL-C ratio after a diet containing mixed CLA isomers (Wanders et al., 2010). A possible explanation for the inconsistencies in these studies may be the differences in doses of CLA used (3.4 - 28 g/d), differences in the study population (overweight and obese versus T2DM) or the effect of different CLA isomers (mixed isomers versus t10,c12 alone).

The individual isomers were shown to have differential effects; t10,12 increased LDL-C : HDL-C and TC : HDL-C compared with c9,t11 (Tricon et al., 2004). CLA also increased concentrations of lipoproteins which have been linked to CVD including increased lipoprotein A compared with placebo (Gaullier et al., 2004, Gaullier et al., 2007) and increased apolipoprotein B after a CLA diet compared with an oleic acid diet (Wanders et al., 2010). Beneficial effects of CLA have been demonstrated for heart rate (Gaullier et al., 2004), blood pressure (Iwata et al., 2007) and platelet aggregation (Sofi

et al., 2010). The latter dietary intervention study is one of few studies which provided a natural source of CLA in the form of 200 g/ week of pecorino cheese. In contrast, Raff *et al* (2006) in another dietary intervention study showed that a diet rich in both CLA isomers (c9,t11 and t10,c12) based on normal butter and an additional 5.5 g of CLA-oil for 5 weeks had no effect on arterial elasticity or blood pressure in healthy young men. Differences in study designs, duration and study populations, as well as concentrations and types of CLA supplementation may account for some of the differences observed in these studies.

Table 1.8 CLA interventions to improve cardiovascular risk profile

Reference	Design	n / subjects	Intervention	Duration	Findings
Gaullier (2004)	DPCT	180/ OW	(1) 4.5 g/d CLA (FFA) or (2) 4.5 g/d CLA (TAG) or (3) 4.5 g olive oil	1 y	(1) ↑ LDL (+0.22 mmol/l); (2) ↓ HDL (-0.09 mmol/l); No Δ between groups
Gaullier (2007)	DPCT	118/ OW & obese	4 g/d CLA / 4.5 g olive oil	6 m	↓ body fat/↓WHR / ↓ HDL-C in CLA
Iwata (2007)	RDPCT	60/ OW men	3.4 g/d CLA or 6.8 g/d CLA or 10.8 g/d safflower oil	12 w	No difference in blood lipids between groups
Moloney (2004)	RDPCT	32/ T2DM	3 g/d CLA / blend palm & soya bean oil	8 w	↑ FG (+0.46 mmol/l)
Raff (2006)	RDB parallel	60/ healthy men	Substitute 115 g fat with butter & 5.5g CLA oil / eucaloric control diet with butter	5 w	No effect on blood pressure
Riserus (2002)	RDPCT	60/ obese	3.4 g/d CLA, or pure t10,c12, or placebo	12 w	t10,c12 ↑ IR (19%) & ↓ HDL (4%)
Tricon (2004)	RDCr	39-49 healthy	3 doses c9,t11 (0.59, 1.19, and 2.38 g/d) or t10,c12 (0.63, 1.26, and 2.52 g/d)	8 w*	t10,c12 ↑ LDL:HDL & TC:HDL. c9,t11 ↓ LDL:HDL & TC:HDL
Sofi (2010)	Crossover	10/ healthy	200 g/week CLA-rich cheese or placebo (regular cheese)	10 w	↓ platelet aggregation (1.1%)
Wanders (2010)	RSMCr	61/ healthy	3 identical diets with 7% energy from (1) CLA mixed isomers, (2) industrial trans fat or (3) oleic acid	9 w	(1) 8.7% ↑ LDL-C; 10% ↑ TC:HDL-C. (2) 11.6% ↑ LDL-C & TC:HDL-C

Δ, change. * 6 week washout. RDPCT, randomised double-blind placebo controlled trial. OW, overweight. CLA, conjugated linoleic acid. w, weeks. DPCT, double-blind placebo-controlled trial. FFA, free fatty acid. TAG, triacylglycerol. y, year. HDL, high density lipoprotein. m, months. WHR, waist:hip ratio. T2DM, type 2 diabetes mellitus. FG, fasting glucose. RDB, randomised double-blind. IR, insulin resistance. RDCr, randomised double blind crossover trial. LDL, low-density lipoprotein. TC, total cholesterol. RSMCr, randomised single blind controlled multiple crossover trial.

1.3.4.3 Cancer

CLA isomers were first shown to inhibit epidermal neoplasia in mice in 1987 and since then the anti-cancer properties of CLA have been extensively investigated (Pariza et al., 2001). CLA has been shown to display anti-cancer effects *in vitro* by a number of mechanisms including inducing apoptosis (Pierre et al., 2013), inhibiting activation of NF- κ B (Rakib et al., 2013) and inhibiting fatty acid synthase (FASN), which is over expressed in carcinoma cells (Song et al., 2012).

In support of these *in vitro* findings, numerous animal studies have identified reduced tumour incidence, metastasis and angiogenesis, as well as anti-oestrogenic effects in response to CLA (Bhattacharya et al., 2006). CLA has also been shown to reduce inflammatory mediators in human lung cancer cells (Oraldi et al., 2013). However, other studies in mice have shown that CLA supplementation promotes tumour progression and carcinogenesis (Meng et al., 2008), and down regulates the expression of adiponectin, leptin and PPAR- γ genes, which have been shown to be protective in the mammary gland (Flowers et al., 2010). Results for the effect of CLA on carcinogenesis are contradictory; t10,c12 has been shown to activate the NF- κ B pathway, which promotes carcinogenesis in a model of intestinal cancer (Rajakangas et al., 2003), but it has also been shown to inactivate NF- κ B resulting in inhibition of human breast cancer cells (Rakib et al., 2013).

Human studies also support the effects of CLA in cancer prevention (**Table 1.9**). Epidemiological data point towards a protective role of foods rich in CLA and reduced risk of colorectal (Larsson et al., 2005) and breast cancer (Aro et al., 2000, Mc Cann et al., 2004), even at 25-year follow up (Knekt et al., 1996). In contrast, CLA was found to either have no association/ no protective effect against breast cancer (Voorrips et al., 2002, Rissanen et al., 2003) or to be negatively associated with breast cancer incidence (Hjartåker et al., 2010). CLA intervention studies in patients with a cancer diagnosis found significantly reduced markers of inflammation (TNF- α and hsCRP), tumour invasion and angiogenesis (matrix metalloproteinase 9) compared with placebo (Mohammadzadeh et al., 2013), as well as decreased breast tumour expression of spot 14, a regulator of FASN (McGowan et al., 2013). However, one study found no association between CLA and prognostic factors, metastasis or death (Chajès et al., 2002). In summary, it appears that CLA has potential cancer preventive effects, but the underlying mechanisms are still being uncovered. Further randomised controlled trials may be necessary to clarify the inconsistencies in the literature.

Table 1.9 CLA and cancer prevention

Reference	Design / cancer	<i>n</i> case / <i>n</i> control	Outcome / intervention	Duration	Findings
Aro (2000)	CS /BrCa	195 / 208	Dietary CLA	3 y	OR for BrCa = 0.4 for highest vs. lowest CLA quintile
Hjartaker (2010)	PC /BrCa	48,844	Childhood & adult milk consumption	6.2y follow-up	Childhood and adulthood milk consumption negatively associated with BrCa
Knekt (1996)	PO /BrCa	4,697	Dairy intake	25y follow-up	Inverse association between milk intake and BrCa
Larsson (2005)	PC/ colorectal	60,708	High fat dairy foods	14.8y follow-up	≥ 4 servings high-fat dairy ↓ risk of colorectal Ca. Each ↑2 servings= 13% ↓ risk
McCann (2004)	CC /BrCa	1,122 / 2,036	Dietary CLA	5y follow-up	Pre-menopausal, highest CLA tertile ↓ risk of ER-negative tumour
Rissanen (2003)	NCC/ BrCa	127 / 242	Serum CLA	10y follow-up	No association between serum CLA and BrCa (samples stored for 25y)
Voorrips (2002)	Cohort/ BrCa	941	Dietary CLA	6.3y follow-up	Weak, positive association between CLA and BrCa
Chajes (2003)	Cohort/ BrCa	209	CLA in Br adipose tissue	7.5y follow-up	No association between CLA and prognostic factors, metastasis or death
McGowan (2013)	OL/ BrCa	24	7.5 g/d 1:1 mixed isomer	Up to 20 d	↓ breast tumour expression of Spot 14
Mohammadzadeh (2013)	RDPCT/ rectal Ca	34	3 g/d CLA (1:1) / placebo (sunflower oil)	5 w	↓ TNF-α (-1.07 pg/ml); ↓ hsCRP (-0.84mg/l); ↓ MMP-9 (-78.1ng/ml)

CS, cross sectional. BrCa, breast cancer. CLA, conjugated linoleic acid. y, years. OR, odds ratio. PO, prospective observational. PC, prospective cohort. CC, case control. ER, estrogen receptor. NCC, nested case control. OL, open label. d, days. RDPCT, randomised double blind placebo controlled trial. w, weeks. Ca, cancer. TNF, tumour necrosis factor. hsCRP, high sensitivity C-reactive protein. MMP, matrix metalloproteinase.

1.3.4.4 Bone health

Numerous *in vitro* studies have demonstrated a beneficial effect of CLA on bone health. c9,t11 has been shown to inhibit osteoclast formation by 70% and a combination of c9,t11 and t10,c12 decreased osteoclast activity by 85-90%, which suggests CLA may inhibit bone resorption (Platt and El-Sohemy, 2009). A recent study found no effect of c9,t11 in murine mesenchymal stem cells, but t10,c12 decreased adipogenesis and increased osteoblastogenesis. Only the effect on adipogenesis was PPAR- γ mediated (Kim et al., 2013a). In animal studies, mice fed a t10,c12-containing diet had higher bone mineral density (BMD) in femoral, tibial and lumbar regions compared with those fed a diet of corn oil and c9,t11 (Rahman et al., 2011). t10,c12 also significantly decreased osteoclastogenic factors in serum of these animals. In mice, CLA in combination with fish oil (Halade et al., 2010) and exercise (Banu et al., 2008) has been shown to significantly increase bone marrow adiposity and bone mass, respectively. The addition of CLA to a diet containing 1% calcium increased bone ash, a surrogate marker for bone mass, compared with mice supplemented calcium only (Park et al., 2011). This effect was only shown for t10,c12 and not c9,t11.

Cross sectional data from postmenopausal women have shown higher than median intakes of CLA to be associated with increased BMD in the forearm (Brownbill et al., 2005). Intervention studies have shown no effect of either 3 g/d or 5 g/d CLA (50:50 mix) for up to 8 weeks on markers of bone formation or resorption in healthy participants (Doyle et al., 2005, Pinkoski et al., 2006). In contrast, another study demonstrated a positive association between red blood cell c9,t11 content, a marker of long-term CLA intake (~4 months), and BMD in men after 16 weeks of supplementation with 3 g/day c9,t11 (Deguire et al., 2012). CLA may be beneficial in increasing osteoblastogenesis, reducing bone marrow adiposity and increasing bone mineral density, but at present we await evidence from human studies to support the *in vitro* and animal results.

1.3.4.5 Body weight

The anti-obesity effect of CLA remains an active area of research over a decade since it was first demonstrated in mice (Park et al., 1997). Human CLA supplementation has been shown to reduce BMI (Gaullier et al., 2005), WC (Riserus et al., 2001) and body fat mass and increase lean muscle mass (Gaullier et al., 2007). A recent systematic review and meta-analysis showed a significant difference in fat loss and BMI favouring the CLA group, but no effect on WC. However, as a result, the authors concluded that CLA had no clinically relevant effect on body composition (Onakpoya et al., 2012). It has been suggested that the unresolved effects of CLA isomers may relate to their antagonistic effects- t10,c12 decreases, while c9,t11 increases adipogenesis (Brown et al., 2003). When the isomers are provided together, the combined effect may counteract the individual effects of each isomer resulting in no significant overall change in adiposity.

A number of studies have investigated the effects of CLA supplementation on body composition. A mix of CLA isomers has been shown to decrease sagittal abdominal diameter (Riserus et al., 2001), waist size (Gaullier et al., 2007) and WHR (Chen et al 2012), while other studies demonstrated no effect on waist size (Gaullier et al., 2007, Carvalho et al., 2012) or body weight (Tricon et al., 2006). Supplementation with a pure t10,c12 isomer was also found to decrease waist girth (Risérus et al., 2002). Skimmed milk (200 ml/d) fortified with 3 g/d mixed CLA isomers was recently shown to decrease body weight and total fat mass in healthy, overweight subjects after 24 weeks (Lopez-Plaza et al., 2013). Another study supplemented healthy subjects with yoghurt-like products enriched with 3.76 g/d mixed CLA isomers for 14 weeks and found no effect on body weight, fat mass or fat free mass, but energy expenditure and PPAR- γ expression were both increased in adipose tissue (Nazare et al., 2007).

The mechanisms underlying the anti-obesity effects of CLA have been investigated extensively and include anti-adipogenic effects, apoptosis and lipolysis and altered fatty acid oxidation and energy expenditure, which are discussed below in more detail.

Anti-adipogenic effects

Reducing TG storage and adipocyte size may be one of the main mechanisms underlying the anti-obesity effects of CLA. t10,c12 has been shown to decrease adipocyte size in rats (DeClercq et al., 2010) and TG accumulation in 3T3-L1

adipocytes (Brown et al., 2001, Evans et al., 2001, Granlund et al., 2003, Miller et al., 2008, den Hartigh et al., 2013). *De novo lipogenesis* was also inhibited by t10,c12 in SGBS adipocytes (Obsen et al., 2012). Contrary to the t10,c12 isomer, c9,t11 has been shown to be less potent in reducing TG accumulation (Fischer-Posovszky et al., 2007) and has been shown to increase adipocyte size in rats (Lopes et al., 2008).

These anti-adipogenic effects of CLA have been suggested to occur as a result of down regulation of PPAR- γ (Kang et al., 2003). A number of studies demonstrated reductions in PPAR- γ , which were associated with reduced lipid accumulation (Brown et al., 2003, Granlund et al., 2003). Brown *et al* (2003) showed that chronic treatment with t10,c12 until day 9 significantly reduced the expression of several adipocyte-specific genes including PPAR- γ , aP2, Plin1 and leptin. In contrast, c9,t11 significantly increased PPAR- γ gene expression in other studies (Brodie et al., 1999, Brown et al., 2004). CLA has also been shown to increase PPAR- γ phosphorylation in an adenosine monophosphate-activated protein kinase (AMP)-dependent manner and to increase the amount of PPAR- γ bound to sirtuin-1, a histone/protein deacetylase that affects energy homeostasis, and is capable of inhibiting PPAR- γ activity (Jiang et al., 2012). Miller *et al* (2008) suggest that t10,c12 may affect the ability of ligands to interact with PPAR- γ , which may explain why in some studies, t10,c12 has been shown not to interact directly with PPAR- γ .

The anti-adipogenic effect of CLA may also occur as a result of alterations in PPAR- γ target genes. Plin1 is a PPAR- γ target gene responsible for protecting the adipocyte from the lipolytic action of cellular lipases. CLA may induce delipidation of adipocytes through down-regulation of Plin1 (Cai et al., 2012). Furthermore, supplementation with 1% t10,12 in mice was shown to significantly decrease Plin1 expression (House et al., 2005). Similar results have been demonstrated in stromal vascular cells from human adipose tissue (Brown et al., 2004) and in primary human adipocytes (Kennedy et al., 2008). In contrast, t10,c12 was also found to increase Plin1 expression, which preceded an increase in lipolysis in primary human adipocytes from abdominal adipose tissue (Chung et al., 2005). Interestingly, c9,t11, a potential PPAR- γ agonist, has been shown to increase Plin1 expression in 3T3-L1 adipocytes (Brown et al., 2003, Zhai et al., 2010). The role of leptin in the anti-adipogenic effects of CLA is unclear; *in vitro* studies show increased leptin secretion in response to CLA (Kim et al., 2013b), while animal studies have shown that a diet containing 1% or 2% CLA significantly reduced

serum leptin in mice (Shelton et al., 2012) and adipose tissue of rats (Gudbrandsen et al., 2009), respectively. However, some studies failed to find an effect of CLA on leptin in animals (Lopes et al., 2008), middle-aged men with PPAR- γ Pro12Ala polymorphism (Rubin et al., 2012) and overweight type 2 diabetics (Shadman et al., 2013).

Apoptosis and lipolysis

In vitro, CLA isomers have been shown to induce apoptosis in human SGBS adipocytes treated with t10,c12 (Fischer-Posovszky et al., 2007). CLA isomers have been suggested to induce lipolysis via alteration of LPL, an enzyme responsible for the hydrolysis of stored TG, but results to support this are inconsistent. In 3T3-L1 adipocytes, t10,c12 has been shown to significantly decrease LPL protein levels (Brown et al., 2003), which occurs via a PPAR- γ -dependent mechanism (Kennedy et al., 2008). In mice fed 1% CLA, adipose LPL mRNA levels decreased (Castellanos-Tapia et al., 2009), while mice fed a high fat diet plus 3% CLA were found to have increased epididymal adipose tissue LPL (Andreoli et al., 2009). Studies in humans do little to clarify these inconsistencies, with some demonstrating reduced LPL mRNA expression in adipose tissue after 16 weeks of supplementation (Raff et al., 2009), while others have shown an increase in plasma LPL in young men prescribed 3 g/d CLA and exercise three times per week (Bulut et al., 2013). Further research is required in the area to determine the exact effects of CLA on LPL-mediated lipolysis. Down-regulation of Plin1 as discussed in the previous paragraph, may also contribute to the lipolytic effects of CLA.

Altered fat oxidation and energy expenditure

Free fatty acid transportation across the outer mitochondrial membrane is a crucial initial step in fatty acid oxidation. Once inside the cell, fatty acids are acylated into fatty acyl-CoA with the help of carnitine palmitoyl transferase 1 (CPT1) (den Hartigh et al., 2013). A recent study in 3T3-L1 adipocytes showed that t10,c12 increased CPT1 and fatty acid oxidation (den Hartigh et al., 2013). Another study in 3T3-L1 adipocytes showed that t10,c12 stimulated fatty acid oxidation and energy expenditure, while c9,t11 had no effect on fatty acid oxidation (Zhai et al., 2010). Similarly, a recent study in mice fed 0.5% of either c9,t11 or t10,c12 showed that t10,c12, but not c9,t11, promoted fatty acid oxidation and thereby improved exercise capacity (Kim et al., 2012). A study in rats fed a 1% mixed CLA diet for 8 weeks showed significant

alterations in proteins involved in fatty acid oxidation and energy dissipation, including PPAR- α , acyl-CoA oxidase and uncoupling protein (Choi et al., 2007). One study in humans has shown that 6 months of supplementation with 4 g/d mixed isomers increased fat oxidation and reduced protein oxidation (Close et al., 2007).

In summary, CLA isomers appear to have differential effects on adipogenesis and fatty acid oxidation; t10,c12 has the most potent anti-obesity effects, while c9,t11 has the opposite effect and may promote adipogenesis by increasing PPAR- γ .

1.3.5 Safety

In some studies, CLA supplementation has been shown to have a negative effect on glycaemic parameters (Moloney et al., 2004) and as a result, concerns have been raised about the safety of CLA supplements given their widespread availability as a weight loss supplement. However, in 2008 the United States Department of Agriculture recognised CLA (Clarinol[®]) as generally recognised as safe for use in certain food types and a European Food Safety Authority assessment in 2010 concluded that consumption of up to 3.75 g/d Clarinol[®] (80% CLA; c9,t11:t10,c12 (1:1)) for up to 6 months had no significant adverse effects on liver function, insulin sensitivity or blood glucose control (EFSA, 2010). In line with these reports, a recent review concluded that supplementation with high quality (80-90%) CLA in the range of 0.5 g - 7 g/day did not cause adverse effects in humans (Dilzer and Park, 2012). Furthermore, a study in patients with T2DM found no effect of CLA on fasting and postprandial glycaemic indicators after 8 weeks (Shadman et al., 2013), and a study in overweight and obese subjects found no effect on glucose metabolism or insulin sensitivity after 6 months with 3.4 g/d CLA (Syvertsen et al., 2007). Interestingly, a recent cross-sectional study found an inverse association between c9,t11 in adipose tissue and risk of diabetes (Castro-Webb et al., 2012).

1.4 Hypothesis

As outlined in the introductory paragraph, the aim of the research described in this thesis was to investigate the contribution of ARVs to adipocyte dysfunction and the role of CLA isomers in mitigating this dysfunction *in vitro*. This research also aimed to explore the prevalence of lipodystrophy in a London-based HIV population, the factors associated with it and the role of CLA supplementation in improving aspects of lipodystrophy *in vivo*. It was hypothesised that CLA isomers would prevent antiretroviral drug-induced adipocyte dysfunction *in vitro* and improve dysregulated body fat distribution, a feature of HALS, *in vivo*.

Chapter 2

Methods

2.1 *In vitro* experiments

2.1.1 General study design

Two sets of *in vitro* experiments were conducted in order to test the hypothesis proposed in this thesis. The first investigated the effect of five different ARVs at physiological concentrations on adipocyte function, gene expression and inflammation. The second set of experiments investigated the effect of two CLA isomers (c9,t11 and t10,c12) on the same markers in adipocytes treated with ARVs. An outline of the study design can be seen in **Figure 2.1**.

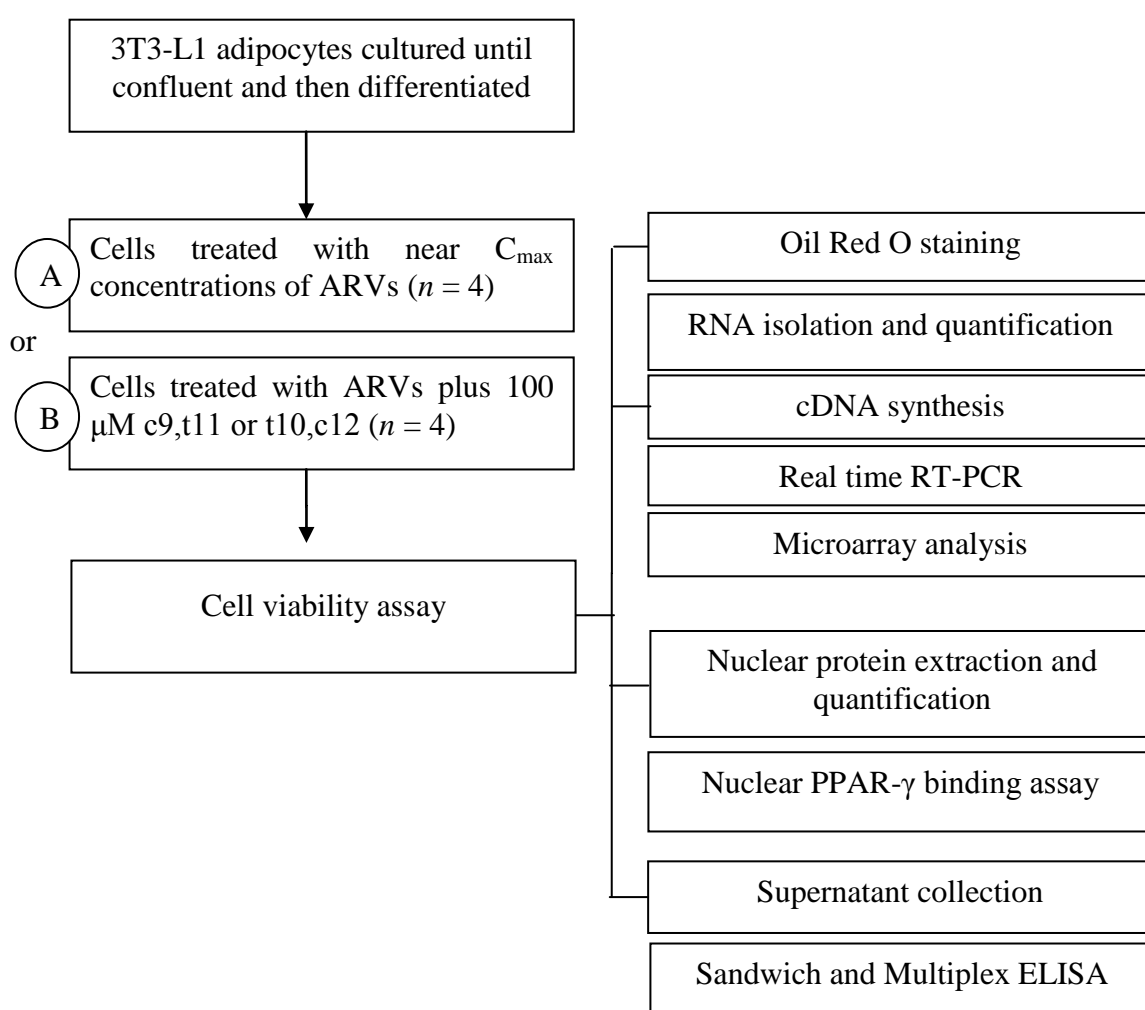


Figure 2.1 *In vitro* study design. 3T3-L1 adipocytes were treated with (A) near C_{\max} concentrations of ARVs with or without (B) 100 μM c9,t11 or t10,c12 for 5 days. Cell viability was confirmed for all treatments prior to experimental work.

2.1.2 Resuscitation of frozen cells and seeding

Cryovials containing 3T3-L1 pre-adipocytes (kind gift of Dr Emilie Stolarczyk, King's College London) were removed from the -80 °C freezer and immediately placed into a 37°C water bath to thaw rapidly. The vials were then wiped with 70% ethanol and transferred to the tissue culture hood. Upon thawing, the entire content of the ampoule was pipetted into 5 ml of pre-warmed DMEM-F12 (Invitrogen Life Technologies, Paisley, UK) supplemented with 10% foetal bovine serum, 8 µg/ml pantothenate (MP Biomedicals Europe, London, UK) and 8 µg/ml biotin, 200 mM L-glutamine, 10,000 U/mL penicillin and 10 µg/L streptomycin (all Sigma Aldrich, Poole, UK) and 10 mg/L MycoKill AB mycoplasma antibiotic (PAA Laboratories Ltd., Yeovil, UK). Cells were centrifuged at 1,000 rpm for 5 minutes. The supernatant was quickly aspirated and the cells resuspended in a 5 ml volume of the same medium. A haemocytometer (Hawksley, Sussex, UK) was used for cell enumeration using a 1:1 mix of the above cell solution with trypan blue stain (Invitrogen Life Technologies) according to manufacturer's instructions. Cells were initially seeded at a density of 0.5×10^6 cells (see **Table 2.1**) in a 75 cm² Nunc[®] tissue culture flask (Thermo Scientific, Roskilde, Denmark) to which the same medium was added giving a final volume of 20 ml.

2.1.3 Routine culture and differentiation

Cells were maintained in an incubator at 37°C with 5% CO₂ until they reached 80-90% confluence after approximately 5 days. Culture medium was carefully removed and the cells were washed twice with pre-warmed 1X phosphate buffered saline (PBS) (Invitrogen Life Technologies, Paisley, PA). A 0.05% v/v solution of Trypsin/EDTA (Sigma Aldrich, Dorset, UK) in PBS was prepared and added to the 75 cm² flask ensuring complete coverage of the cell monolayer. The flask was incubated until the cells detached after 2 minutes. The trypsin/EDTA solution was quenched by the addition of 20 ml of fresh pre-warmed medium. The cell solution was centrifuged at 1,000 rpm for 5 minutes. The supernatant was removed and the cells were resuspended in 5 ml of medium. Cells were counted as described previously and seeded in 6-, 24- or 96- well plates for experimental work as shown in **Table 2.1**.

Table 2.1 3T3-L1 seeding densities

Plate/flask type	Total volume of medium per well	Seeding density per well	Total seeding density per plate
75 cm ² flask	20 ml	0.5×10^6	0.5×10^6
6-well plate	3 ml	4×10^4	2.4×10^5
24-well plate	500 μ l	2.5×10^3	0.6×10^5
96-well plate	100 μ l	1.56×10^2	0.15×10^5

2.1.4 Adipocyte differentiation

Cells were incubated for a further 48 hours to initiate growth arrest. On day 7, pre-adipocyte medium was replaced with medium supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) (Cayman chemical, Cambridge Biosciences, Cambridge, UK), 0.25 μ M dexamethasone (Sigma Aldrich, Dorset, UK) and 1 μ g/ml insulin (Sigma Aldrich, Dorset, UK) (Differentiation medium). **Table 2.2** illustrates the different media used for maintenance, differentiation and treatment of 3T3-L1 adipocytes in this study.

Table 2.2 Media used to maintain and induce differentiation of 3T3-L1 adipocytes

Pre-adipocyte (maintenance) medium	Differentiation medium	Treatment medium
500 ml DMEM-F12	Pre-adipocyte medium in necessary volume	Pre-adipocyte medium in necessary volume
10% foetal calf serum	1 μ g/ml insulin	1 μ g/ml insulin
200mM L-glutamine penicillin / streptomycin	0.25 μ M dexamethasone	ZDV (1 μ M) / d4T (10 μ M) / RTV (20 μ M) / IDV (10 μ M) / TDF (1 μ M)
10 μ g/ml MycoKill AB mycoplasma antibiotic	0.5 mM 3-isobutyl-1- methylxanthine	100 μ M c9,t11 / t10,c12
8 μ g/ml biotin		
8 μ g/ml pantothenate		
DMEM-F12, Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12. ZDV, zidovudine. d4T, stavudine. RTV, ritonavir. IDV, indinavir. TDF, tenofovir.		

2.1.5 Adipocyte treatment

After two days (day 9), medium was removed and cells were washed with pre-warmed PBS. Medium was replaced with treatment medium (**Table 2.2**) containing ZDV, d4T, RTV, IDV or TDF at concentrations comparable to their C_{\max} in patients' serum, and 100 μM CLA isomers (c9,t11 or t10,c12). The vehicle for ARVs was ethanol (filter sterilised; VWR, Leicestershire, UK) and for CLA it was DMSO. In all experiments, all treated cells received one or both vehicles consisting of 0.1% ethanol and/or 0.1% DMSO as appropriate. Medium was changed every 2 days until cells were fully differentiated at day 14. Cells were exposed to the respective treatments for a total of five days.

2.1.6 Preparation of antiretroviral drugs

Ethanol was used as the vehicle for the ARVs used in these *in vitro* experiments. ARV stock solutions (1000X) were prepared, aliquoted and stored at $-20\text{ }^{\circ}\text{C}$ prior to use. ARVs were diluted in differentiation medium to near C_{\max} concentrations (the maximum drug concentration observed in patients' serum) as outlined in **Table 2.3**.

Table 2.3 Preparation of antiretroviral drugs

Drug	Drug class	Supplier	Mw	Stock conc.	C_{\max} conc.
ZDV	NRTI	Sigma Aldrich	267.24	1 mM	1 μM
d4T	NRTI	Sigma Aldrich	224.21	10 mM	10 μM
RTV	PI	Santa Cruz Biotechnology Inc.	720.94	20 mM	20 μM
IDV	PI	Santa Cruz Biotechnology Inc.	711.87	10 mM	10 μM
TDF	NtRTI	Santa Cruz Biotechnology Inc.	287.21	1 mM	1 μM

Mw, molecular weight. ZDV, zidovudine NRTI, nucleoside reverse transcriptase inhibitor. d4T, stavudine. RTV, ritonavir. PI, protease inhibitor. IDV, indinavir. TDF, tenofovir. NtRTI, nucleotide reverse transcriptase inhibitor.

2.1.7 Preparation of CLA isomers

DMSO was used as the vehicle for CLA isomers in these experiments. Stocks solutions of 100 μ M of each CLA isomer were prepared as shown in **Table 2.4** and 20 μ l aliquots stored at -80 °C prior to use. Each CLA isomer was prepared to a working concentration of 100 μ M in cell culture medium. The working concentration of c9,t11 added to cells is four-fold greater than that observed in plasma of healthy subjects, while that of t10,c12 is 28-fold greater than *in vivo* concentrations (Shafer, 2006).

Table 2.4 Preparation of fatty acid stock solutions

Isomer	Supplier	Mw	Stock conc. (100 mmol/l)	Working conc. (100 μ mol/L)
<i>trans</i> -10, <i>cis</i> -12	VWR International	280.5	28.05 mg/ml	28.05 mg/L
<i>cis</i> -9, <i>trans</i> -11	VWR International	280.5	28.05 mg/ml	28.05 mg/L

Mw, molecular weight.

2.1.8 Cell viability

A colorimetric cell viability assay was used in determining the number of viable cells in culture. The CellTiter 96[®] AQueous One Solution Reagent (Promega, Southampton, UK) contains 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and an electron coupling reagent (phenazine ethosulfate; PES). Prior to conducting the MTS assay, cells were washed with pre-warmed medium three times. 100 μ l of medium was then added to each well followed by 20 μ l of MTS assay solution. Cells were incubated for 1 h at 37 °C with 5% CO₂. The quantity of formazan produced by cellular reduction of MTS is directly proportional to the number of living cells in culture and was measured at 490 nm in a 96-well plate reader (Synergy HT-1, Biotek, Bedfordshire, UK).

2.1.9 Oil Red O staining

Oil Red O (ORO) stain is a lysochrome fat soluble diazo dye used to stain neutral lipids (Sigma Aldrich, 2014a). The stain was used to indicate the degree of adipogenesis in differentiated adipocyte cultures. ORO stock solution was prepared as follows: 0.7g of ORO powder (Sigma Aldrich, Dorset, UK) was dissolved in 200 ml of propan-2-ol (VWR International, Leicestershire, UK). This mixture was stirred overnight using a magnetic stirrer, filtered through a 0.22 μ m membrane filter (Nalgene[®] Thermo

Scientific, Fischer Scientific Ltd. Leicestershire, UK) and stored at 4°C until use. To make the ORO working solution six parts of ORO stock solution were added to 4 parts distilled water. This solution was mixed, allowed to sit at room temperature for 20 minutes and then filtered through a 0.22 µm membrane filter. Induction medium was removed from the plate and cells were washed once with PBS. 500 µl of 10% formalin (Sigma Aldrich, Dorset, UK) was added to each well and cells were incubated at room temperature for 5 minutes. The formalin was discarded and the same volume of fresh formalin added. Parafilm and aluminium foil were placed around the plate to prevent it drying out and the plate was incubated at room temperature for 1 hour. After this time, the formalin was removed and cells in each well were washed with 500 µl of 60% propan-2-ol then allowed to dry completely. 200 µl of ORO working solution was added to each well, taking care not to touch the walls of the wells, and cells were incubated for 10 minutes at room temperature. All of the ORO was removed and distilled water was added immediately. Cells were washed four times with distilled water. The distilled water was then removed and cells were allowed to air dry completely. Photographs of cell morphology were taken with a DXM 1200 Digital Camera (Nikon, Kingston Upon Thames, UK) and a Lucia G Image-Processing System (version 4.61; Nikon). The ORO was eluted by adding 750 µl of 100% propan-2-ol to each well and cells were then incubated at room temperature for 10 minutes. The ORO and propan-2-ol were pipetted up and down several times to ensure that all of the ORO was in solution. 250 µl of this solution was then transferred to wells of a 96-well plate and the optical density of the solution was measured at 540 nm in a spectrophotometer (Synergy HT).

2.1.10 RNA extraction

Total RNA was extracted from adipocytes on day 14 using the RNeasy Mini Kit (Qiagen, Manchester, UK). Cells were lysed directly in each well by adding 350 µl proprietary Buffer RLT, a highly denaturing guanidine-thiocyanate-containing buffer which inactivates RNases to ensure purification and supports the binding of the RNA to the silica membrane. Lysate was collected using a pipette and pipetted into a microcentrifuge tube. Lysate was vortexed or mixed to ensure no cell clumps were visible. 350 µl of 70% ethanol was added to the homogenised lysate to provide appropriate binding conditions, and mixed well by pipetting. Up to 700 µl of the sample was transferred, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube. The lid was closed gently, and the tube centrifuged for 15 seconds at 10,000 rpm. Total RNA bound to the membrane and

contaminants were washed away as the flow-through was discarded. 700 µl buffer RW1 was added to the RNeasy spin column. Buffer RW1 is a washing buffer containing a guanidine salt, as well as ethanol, used for the removal of biomolecules such as carbohydrates that are non-specifically bound to the membrane. The lid was closed gently, and the tube centrifuged for 15 seconds at 10,000 rpm to wash the spin column membrane. The flow-through was discarded. 500 µl Buffer RPE was added to the RNeasy spin column. Buffer RPE is a mild washing buffer containing ethanol, which removed traces of salts associated with use of previous buffers. The lid was closed gently, and the tube centrifuged for 15 seconds at 10,000 rpm to wash the spin column membrane. The flow-through was discarded. 500 µl Buffer RPE was added to the RNeasy spin column. The lid was closed gently, and the tube centrifuged for 2 min at 10,000 rpm to wash the spin column membrane. The long centrifugation dried the spin column membrane, ensuring that no ethanol was carried over during RNA elution. Residual ethanol interferes with downstream reactions. The RNeasy spin column was placed in a new 1.5 ml collection tube. 30 µl RNase-free water was added directly to the spin column membrane. The lid was closed gently, and the tube centrifuged for 1 min at 10,000 rpm to elute the RNA.

2.1.11 RNA quantification

The quality of the RNA was assessed using the NanoDrop[®] 1000 3.7.1 spectrophotometer (Thermo Scientific, USA). The NanoDrop[®] accurately measures RNA quantity and integrity up to 3,700 ng/µl without dilution. It automatically detects the high concentration and utilises the 0.2 mm path length to calculate absorbance and determine RNA concentration in ng/µl as shown in **Table 2.5**. For NanoDrop[®] spectrophotometry, 1.0 µl of sample was used to quantify RNA concentration. A blank of nuclease-free water was used to optimise the NanoDrop[®]. Sample pedestals were first cleaned with nuclease-free water and ethanol prior to use.

Table 2.5 RNA quality analysis using the NanoDrop[®]

Sample ID	User ID	Date	Time	ng/ul	A260	A280	260/280	260/230	Constant	Cursor Pos.	Cursor abs.	340 raw
F12	Default	9/24/2013	3:54 PM	-32.53	-0.651	0.274	-2.38	-0.12	50.00	230	5.219	0.048
F9	Default	9/24/2013	3:56 PM	42.92	0.858	0.479	1.79	0.97	50.00	230	0.882	0.053
F2	Default	9/24/2013	3:59 PM	105.89	2.118	1.147	1.85	1.62	50.00	230	1.304	0.107
G1	Default	9/24/2013	4:00 PM	66.71	1.334	0.705	1.89	1.11	50.00	230	1.197	0.138
E9	Default	9/24/2013	4:00 PM	26.85	0.537	0.279	1.93	0.84	50.00	230	0.638	-0.072
E7	Default	9/24/2013	4:01 PM	35.21	0.704	0.396	1.78	1.19	50.00	230	0.591	0.047
E6	Default	9/24/2013	4:02 PM	31.38	0.628	0.365	1.72	0.75	50.00	230	0.841	0.046
E2	Default	9/24/2013	4:03 PM	66.78	1.336	0.774	1.72	0.90	50.00	230	1.477	0.054

The ratio of absorbance at 260 and 280 nm (260/280) was used to assess the integrity of the RNA. A ratio of ~2.0 is generally accepted as pure for RNA. A lower ratio may indicate the presence of contaminants such as protein or phenol that absorb strongly at or near 280 nm. After quantifying the RNA and assessing the integrity, samples were aliquoted and frozen at -80°C. Samples were defrosted as required for analysis.

2.1.12 Reverse transcription

During reverse transcription, a DNA polymerase enzyme (reverse transcriptase) incorporates deoxynucleotides sequentially along a single-stranded RNA template strand and catalyses the formation of phosphodiester bonds between these nucleotides to generate a complementary DNA (cDNA) strand. *In vitro*, DNA polymerase generates complementary strands using a 3' hydroxyl group provided by an oligonucleotide primer (Avison, 2007). The result is a labelled cDNA strand complementary to the target RNA species of interest. The number of cDNA target molecules equals the number of RNA molecules copied and provided the RT reaction goes to completion, all reagents are in excess and each RNA molecule is copied only once, the RNA molecule of interest can be quantified (Avison, 2007).

cDNA fragments were generated by reverse transcription using the High Capacity RNA-to-cDNA kit (Invitrogen Life Technologies). Up to 2 µg of total RNA was used per 20 µl reaction. Kit components were thawed on ice. Up to 9µl of each RNA sample was added to a microcentrifuge tube (Starlab, Milton Keynes, UK). To this, 10 µl reverse transcriptase buffer was added along with 1 µl reverse transcriptase enzyme mix (Invitrogen Life Technologies). This volume was made up to 20µl with nuclease-free water. Microcentrifuge tubes were sealed, mixed gently and centrifuged (Jencons, Bedfordshire, UK) to spin down the contents and eliminate air bubbles. Tubes were placed in a thermal cycler (Thermo Electron Corporation, Thermo Scientific) set at conditions optimised for use with the High Capacity RNA-to-cDNA kit as shown in **Table 2.6**.

Table 2.6 Reverse transcription conditions

	Step 1	Step 2	Step 3
Temperature (°C)	37	95	4
Time	60 min	5 min	∞

2.1.13 Real-time reverse transcriptase polymerase chain reaction

Real-time reverse transcriptase polymerase chain reaction (RT-PCR) is a well established, mainstream technology for the detection and quantification of RNA targets (Bustin and Mueller, 2005). The discovery of Taq polymerase in the early 1980s eliminated the need for the addition of a new enzyme previously destroyed during thermocycling (Avison, 2007). The Taq polymerase has a 5'–3' exonuclease activity, as well as dual-labelled fluorogenic oligonucleotide probes. The probes emit a fluorescent signal only upon cleavage. In this system, a probe (TaqMan[®]) is designed to anneal to the target sequence between the classical forward and reverse primers (**Figure 2.2**). The probe is labelled with both a reporter fluorochrome (e.g., 6-carboxyfluorescein, or FAM) at one end and a quencher dye (e.g., 6-carboxy-tetramethyl-rhodamine, or TAMRA) at the 3' end. In its intact form, the fluorescence emission of the reporter dye will be absorbed by the quencher dye. The melting point of the probe is 10°C higher than that of the primers in order to anneal to the amplicon during the extension phase of the PCR process (performed at 60°C). The probe is then degraded during the extension phase by the 5'–3' exonuclease activity of the Taq polymerase. The reporter and quencher are separated, which results in an increase in the fluorescence emission of the reporter. Fluorescence released is directly proportional to the amount of product generated in each PCR cycle and thus can be applied as a quantitative measure of the PCR product formed (Avison, 2007).

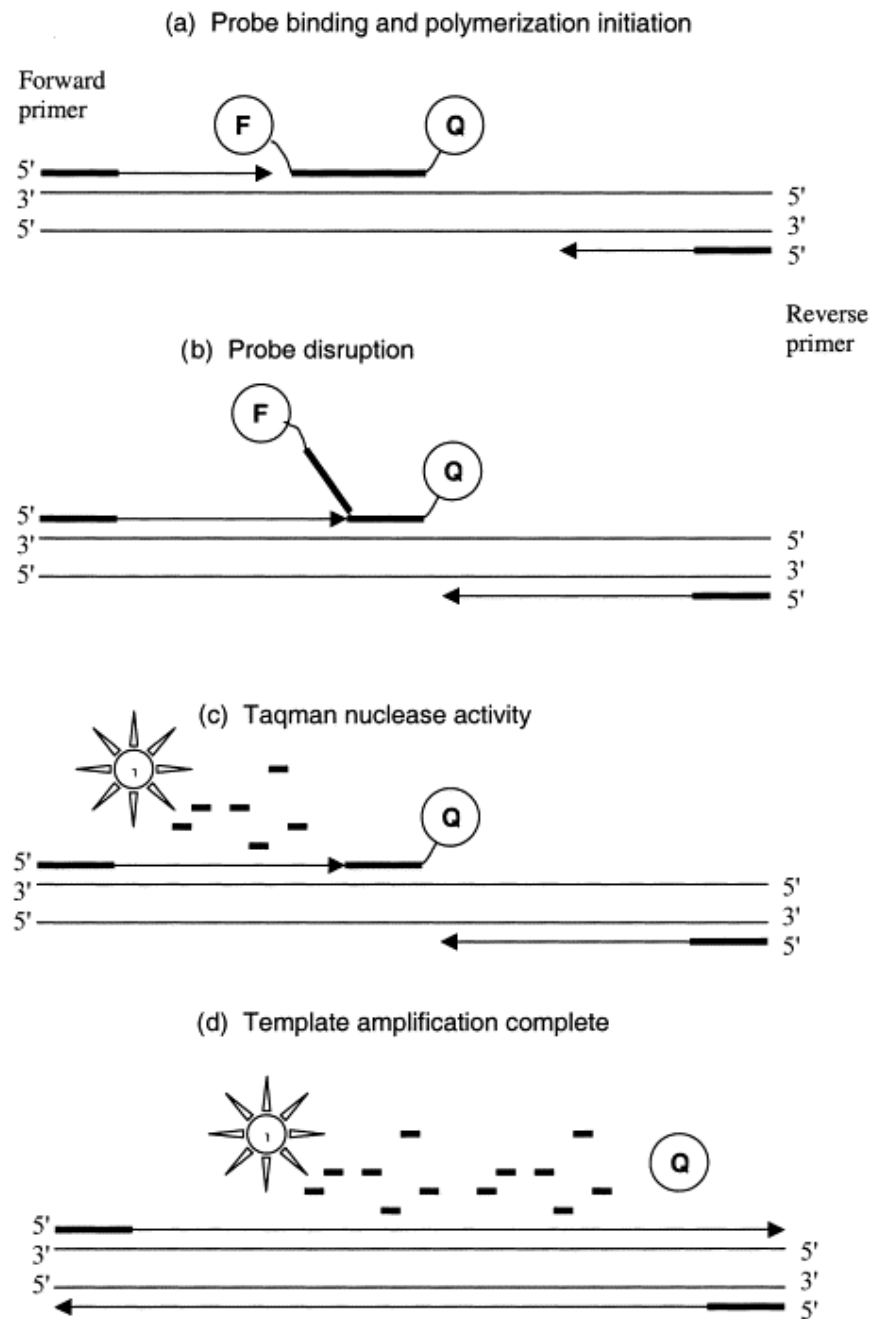


Figure 2.2 Taqman[®] RT-PCR system (*Reproduced from (Epstein et al., 2002)*)

cDNA (1 µg) was used in real-time RT-PCR to measure PPAR-γ, adiponectin, leptin, resistin, IL-6, perilipin and Cidea gene expression, and 1 µg of cDNA was used in the hypoxanthine-guanine phosphoribosyltransferase (Hprt) control RT-PCR. Reactions were prepared in a MicroAmp™ Optical 96-well reaction plate (Invitrogen Life Technologies) to give a total volume of 25 µl. PCR reaction mix components and volumes are detailed in **Table 2.7**. cDNA template was added to each well followed by TaqMan® gene expression assays (20X) specific for each target gene. TaqMan® gene expression assays consist of a pair of unlabelled PCR primers and a TaqMan® probe with a FAM™ or VIC® dye label on the 5' end and minor groove binder (MGB) and non-fluorescent quencher (NFQ) on the 3' end. TaqMan® Gene Expression Master Mix (2X) was also added, which consists of AmpliTaq Gold® DNA polymerase, a passive internal reference based on proprietary ROX™ dye and AmpErase® Uracil N-Glycosylase. Diethylpyrocarbonate (DEPC)-treated water was added to make up a total volume of 25 µl in each well. Each plate also contained a no template control (NTC) containing all reaction components except the cDNA template. A no-reverse transcriptase (RT) control containing all reaction components except the TaqMan® reverse transcriptase was also added to each plate. DEPC-treated water was used to replace the cDNA template and reverse transcriptase in the NTC and no-RT controls, respectively. The PCR plate was covered with a MicroAmp™ Optical Film and centrifuged briefly in a PCR Plate Spinner (Labnet International, Edison, USA) to spin down the contents and eliminate air bubbles. A MicroAmp™ Optical Film Compression Pad was applied to the top of the plate and the plate placed in the ABI Prism 7000 Sequence Detection System (Invitrogen Life Technologies). Thermal cycling conditions were set to default PCR thermal cycling conditions as shown in **Table 2.8**.

Table 2.7 RT-PCR reagents and volumes

Sample	Reaction mix component	Volume per 25 µl reaction
No template control	TaqMan [®] Gene Expression Master Mix (2X)	12.5
	TaqMan [®] Gene Expression Assay	1.25
	DEPC-treated water	11.25
No-RT control	TaqMan [®] Gene Expression Master Mix	12.5
	cDNA template	2.00
	DEPC-treated water	10.5
Samples	TaqMan [®] Gene Expression Master Mix	12.5
	TaqMan [®] Gene Expression Assay	1.25
	cDNA template	2.00
	DEPC-treated water	9.25

DEPC, diethylpyrocarbonate. RT, reverse transcriptase.

Table 2.8 RT- PCR conditions

	Step	Time	Temperature
UDG Incubation	HOLD	2 min	50 °C
Taq Enzyme activation	HOLD	10 min	95 °C
PCR (40 cycles)	Denature	15 sec	95 °C
	Anneal/ extend	1 min	60 °C

UDG, uracil DNA glycosylase

2.1.14 Global gene expression studies

2.1.14.1 RNA amplification and labelling

Previously quantified RNA samples extracted using the RNeasy Mini Kit (Qiagen) were pooled and 500 ng of each pooled sample in 5 µl nuclease-free water was used in the reactions. The MessageAmp™ Premier RNA Amplification kit (Invitrogen, UK) was used for cDNA synthesis using ArrayScript™ reverse transcriptase. cDNA was then used in an in-vitro transcription (IVT) reaction to synthesise biotin-modified amplified RNA (aRNA) according to manufacturer's protocol. The kit employs the reverse transcription T7 in vitro transcription (RT-IVT) method, considered the gold standard for sample preparation in micro-array profiling. After synthesis, the biotin-modified aRNA was purified to remove enzymes, salts and unincorporated nucleotides using the same kit. The concentration of aRNA was determined using the NanoDrop® spectrophotometer (Thermo Scientific). Biotinylated aRNA was fragmented prior to hybridisation using the 5X Array Fragmentation Buffer supplied with the MessageAmp™ Kit.

2.1.14.2 aRNA fragmentation and hybridisation

The sample was then evaluated using the Agilent®2100 Bioanalyser instrument (Agilent Technologies Inc., Santa Clara, USA) and RNA 6000 Nano Kit loaded with 300 ng of aRNA per well. The fragmented aRNA had a distribution of 35-200 nucleotides with a peak at approximately 100-200 nucleotides. **Figure 2.3** shows typical bioanalyser electropherograms of unfragmented and fragmented aRNAs. 11 µg of aRNA were hybridised onto the Affymetrix Mouse Genome 430 2.0 Array Kit (Affymetrix, High Wycombe, UK), which covers over 39,000 transcripts on a single array of the mouse genome. The arrays were subsequently washed, stained and scanned on the Fluidics Station 400 and the GeneArray Scanner (Agilent Technologies, Inc.) according to the manufacturer's protocol.

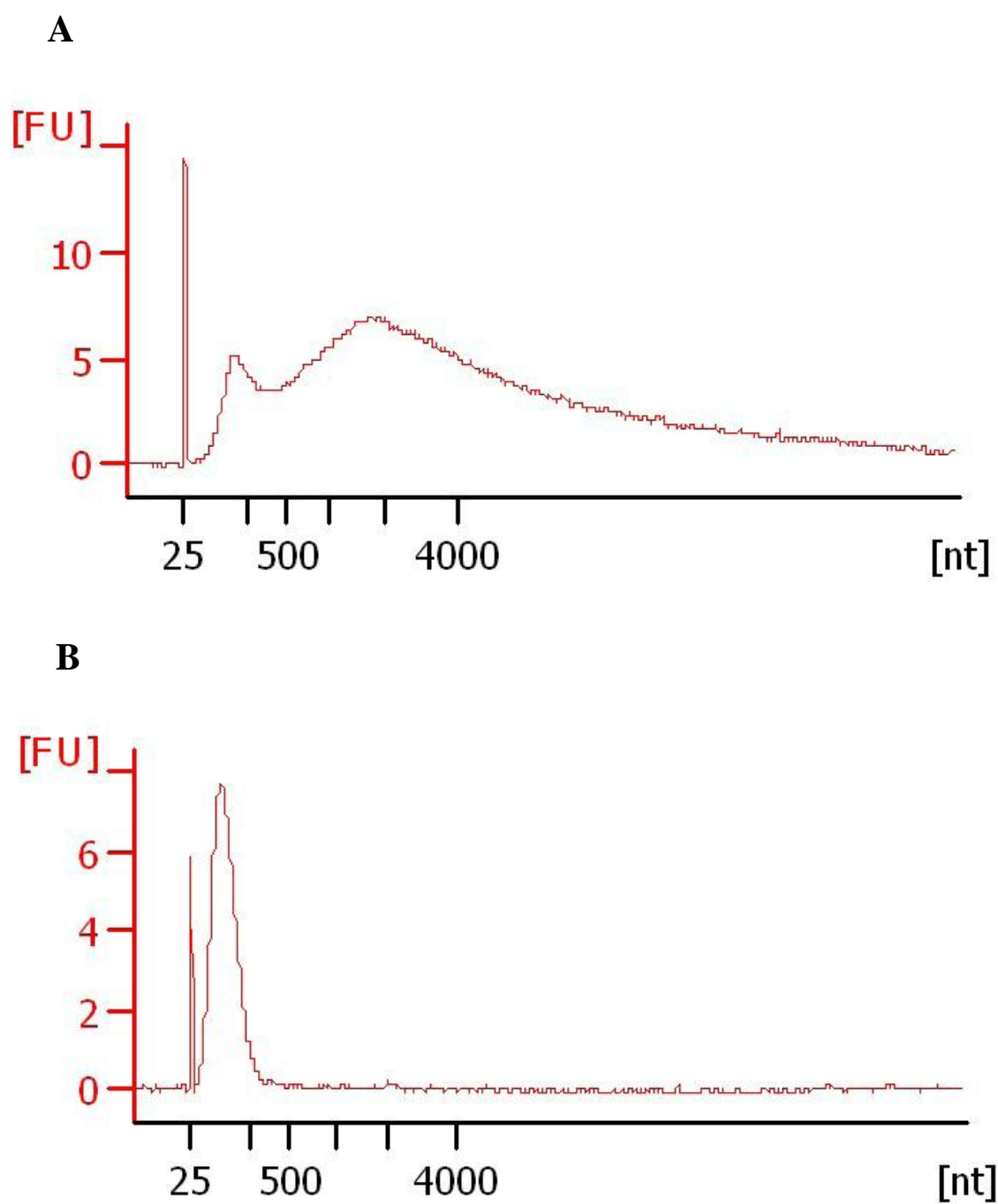


Figure 2.3 Typical bioanalyser electropherograms of (A) unfragmented and (B) fragmented aRNAs

2.1.14.3 Microarray data analysis

The Robust Multi-array Average approach was used to analyse the data using quantile normalisation and the average change in expression was determined for each gene using the formula $\text{=IF}(\text{'ratio value'} < 1, -1/\text{'ratio value'}, \text{'ratio value'})$ where the ratio value is ratio of treatment/control. A two-fold change relative to control (signal log ratio ≥ 1.0 or ≤ -1.0) was selected as the threshold to define differentially expressed genes. Altered gene lists were imported into MetaCore™ pathway analysis software (GeneGo, Thomson Reuters, New York) where differentially expressed genes were classified into functionally-related gene clusters and signalling and metabolic pathway maps were generated.

2.1.15 Enzyme-linked immunosorbent assay

Enzyme-linked Immunosorbent Assay (ELISA) is a fundamental technique in cell biology used to detect and colorimetrically quantitate the presence of antigen (AbD Serotec, 2014). Commercially available ELISA kits employ a quantitative sandwich enzyme immunoassay technique, which allows for the measurement of antigen in cell culture supernatants. A primary (capture) antibody is immobilised to a 96-well microplate and the sample and standards are sandwiched to this antibody. The secondary (detection) antibody is conjugated with the enzyme horseradish peroxidase (HRP), which acts upon a substrate tetramethylbenzidine (TMB), to produce a blue colour directly proportional to the amount of antigen present. An acidic solution such as H_2SO_4 , stops the reaction and produces a yellow colour change (**Figure 2.4**).

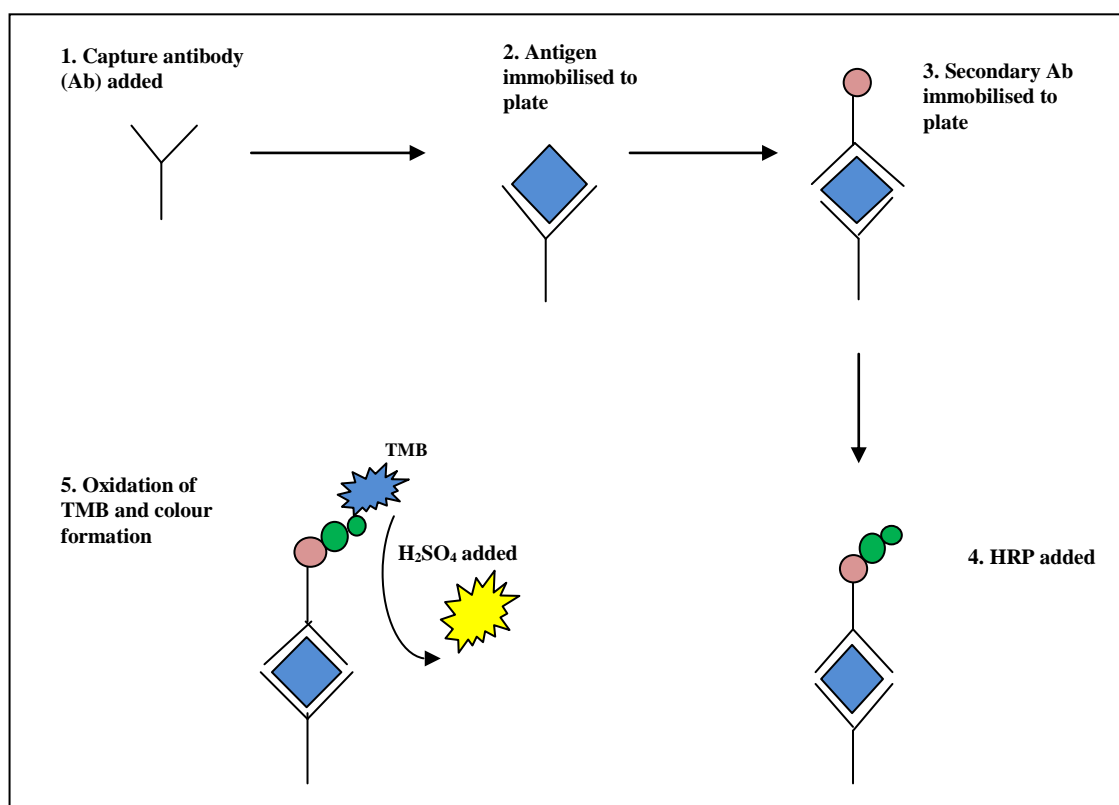


Figure 2.4 Principles of sandwich ELISA

2.1.15.1 Mouse Adiponectin ELISA

The commercially available DuoSet ELISA Development kit (R&D Systems, Abingdon, Oxfordshire, UK) was used for the capture of antigen in samples of cell supernatants. This kit included primary (capture) antibody, secondary (detection) antibody, standard and streptavidin-HRP, and was stored at 4°C. Capture antibody, detection antibody and standards were supplied as lyophilised powders and were reconstituted and stored at -20 °C until use. Prior to performing the assay of the complete sample set, a preparatory ELISA was performed to establish optimal dilution factors for the supernatants, to ensure they fit within the sensitivity of the kit. Typical adipokine concentrations published from the 3T3-L1 cell line were reviewed. Supernatant samples were diluted according to expected adipokine yield and the sensitivity of the kit. Capture antibody was diluted to a working concentration, as per the manufacturer's instruction, with PBS and coated onto a microtitre plate (StarLab). The plate was sealed and incubated overnight at room temperature. The plate was then washed by submerging in 0.05% v/v Tween 20[®] (Sigma Aldrich) in PBS, gently agitating and flicking out the wash buffer. This was performed three times. Plates were

then tapped against paper towel to dry. Wells were blocked with reagent diluent (1% w/v bovine serum albumin (BSA; Sigma Aldrich) in PBS) for 1 hour. Standards were reconstituted to working concentrations using serial 1:2 dilutions in reagent diluent. Samples were diluted to optimal concentrations as determined in the optimisation step in reagent diluent. After blocking with BSA, wells were washed as before and loaded with standards, samples and blank wells consisting of reagent diluent alone. Plates were sealed and incubated at room temperature for 2 hours. Wells were washed as before and loaded with detection antibody for 2 hours. The plate was washed three times and the streptavidin-HRP enzyme was added and allowed to sit at room temperature and away from direct light for 20 minutes. Plates were washed again. TMB substrate solution (Sigma Aldrich) was added to each well and the plate incubated at room temperature for 20 minutes to oxidise the TMB. Stop solution (2N H₂SO₄) was added, which stopped the reaction, causing a colour change from blue to yellow. Absorbance was read in a microplate reader (Synergy HT) at 450 nm with wavelength correction at 570 nm. A standard curve was constructed by plotting the mean absorbance values (optical density) from the standard solutions versus the known adipokine concentrations. Sample concentrations were calculated using the equation of the standard curve.

2.1.16 Multiplex ELISA

A MILLIPLEX™ MAP Mouse Adipocyte Panel (Merck Millipore, Watford, UK) was used for the simultaneous quantification of leptin, resistin and IL-6 in cell culture supernatant samples. The MILLIPLEX™ MAP system is based on the Luminex® xMAP® technology, which internally colour-codes microspheres with two fluorescent dyes. Through precise concentrations of these dyes, 100 distinctly coloured specific capture antibody-immobilised bead sets are created. After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced. The reaction mixture is then incubated with Streptavidin-PE conjugate (PE), the reporter molecule, to complete the reaction on the surface of each microsphere. The microspheres pass rapidly through a laser, which excites the internal dyes marking the microsphere set. A second laser excites PE, the fluorescent dye on the reporter molecule. Finally, high-speed digital-signal processors identify each individual microsphere and quantify the result of its bioassay based on fluorescent reporter signals. Cell supernatants from treated adipocytes were collected on day 14, centrifuged to remove debris, aliquoted and stored at -20°C. Prior to the assay, all reagents were warmed to room temperature. The

assay was performed in duplicate. Standards were prepared from serial dilutions of the 50,000 pg/ml highest standard as shown in **Table 2.9**.

Table 2.9 Preparation of working standards for multiplex ELISA

Standard concentration (pg/ml)	Volume of assay buffer (µl)	Volume of standard (µl)
50,000	-----	-----
12,500	150	50 µl of 50,000
3,125	150	50 µl of 12,500
781.3	150	50 µl of 3,125
195.3	150	50 µl of 781.3
48.8	150	50 µl of 195.3
12.2	150	50 µl of 48.8
3.2	150	50 µl of 12.2

The Microtiter Filter Plate was set on a plate holder so that the bottom of the plate did not touch any surface. The filter plate was pre-wet by pipetting 200 µl of the proprietary assay buffer into each well of the plate. The plate was sealed and mixed on a plate shaker for 10 minutes at room temperature (20-25°C). A plate vacuum was used to remove the assay buffer taking care not to invert the plate. Excess buffer was removed by blotting the bottom of the plate with a paper towel. 10 µl of each standard or control was added to appropriate wells. Assay buffer was used as the background for the 0 pg/ml standard. 10 µl assay buffer was added to each sample well followed by 10 µl of appropriate adipokine-free medium to the background, standards, and control wells and 10 µl of sample into the appropriate wells. The mixing bottle containing the antibody-immobilised beads was vortexed and 25 µl of the mixed beads was added to each well. The plate was sealed and covered with a lid. A rubber band was wrapped around the plate holder, plate and lid and incubated with agitation (500-800 rpm) on a plate shaker over night at 4°C. After the overnight incubation, the fluid was gently removed by vacuum, taking care not to invert the plate. The plate was then washed 3 times with 200 µl/well of wash buffer, removing wash buffer by vacuum filtration between each wash. Excess buffer was removed by blotting the bottom of the plate with a paper towel. 50 µl detection antibodies were added to each well. The plate was sealed, covered with a lid and incubated with agitation on a plate shaker for 30 minutes at room temperature. After the incubation period, 50 µl PE was added to each well containing the detection antibodies. The plate was sealed again, covered with a lid and incubated with agitation on a plate shaker for 30 minutes at room temperature. After the incubation, the contents

of each well were removed gently by vacuum taking care not to invert the plate. The plate was washed 3 times with 200 µl/well wash buffer. Wash buffer was removed by vacuum filtration between each wash. Excess buffer was removed by blotting the bottom of the plate with a paper towel. 100 µl of sheath fluid was added to all wells and the beads resuspended on a plate shaker for 5 minutes. The Luminex 100™ IS (Merck Millipore, Watford, UK) was set up according to assay protocol as shown in **Table 2.10**. Data for the Median Fluorescent Intensity (MFI) using a weighted 5-parameter logistic was used to calculate analyte concentrations of standards and samples.

Table 2.10 Luminex 100™ IS settings

Events	50 per bead	
Sample size	50 µl	
Gate settings	8,000 to 15,000	
Reporter Gain	Default (low PMT)	
Time Out	60 seconds	
Bead Set	<u>Beads</u>	<u>Luminex Bead Region</u>
	Leptin	16
	IL-6	58
	Resistin	78

IL-6, interleukin-6. PMT, photo multiplying tubes.

2.1.17 Nuclear protein extraction

The Active Motif Nuclear Extract kit (Active Motif, Rixensart, Belgium) was used for the extraction of nuclear proteins from adipocytes on day 14 as per manufacturer's protocol. Briefly, cells were washed with ice-cold PBS/phosphatase inhibitors. This solution was aspirated out and cells removed from the plate by gently scraping with a plastic Pasteur pipette. Cells were centrifuged at 500 rpm for 5 minutes, the supernatant discarded and pellet gently resuspended in 500 µl of proprietary hypotonic buffer. The suspension was incubated for 15 minutes on ice. Detergent (10% NP-40) was added and the suspension was vortexed for 10 seconds. The suspension was then centrifuged for 30 seconds at 13,000 rpm and the supernatant (cytoplasmic fraction) transferred into a pre-chilled microcentrifuge tube (Starlab), aliquoted and stored at -80°C. The pellet was then resuspended in complete lysis buffer and incubated on ice for 30 minutes on a rocking platform (Bibby Scientific, Stone, UK). The pellet was vortexed and then centrifuged for 10 minutes at 13,000 rpm. The supernatant (nuclear fraction) was then transferred into pre-chilled microcentrifuge tubes, aliquoted and stored at -80°C.

2.1.17.1 Protein quantification

The Bradford protein assay is one method used to determine total protein concentration in a sample. Proteins present in solution form a complex with the Bradford Reagent dye (containing Brilliant Blue G; Sigma Aldrich) and the resulting protein-dye complex shifts the absorption maximum of the dye from 465 nm to 595 nm. The amount of absorption is proportional to the protein present in solution. The Bradford reagent is compatible with low concentrations of detergents present in buffers frequently used to stabilise proteins in solution. For nuclear extracts, samples were diluted 1:2 in PBS giving a total volume of 5 µl per well. Standards were prepared from a stock solution of 2 mg/ml BSA (Sigma Aldrich) providing a linear standard concentration range of between 0-2 mg/ml in PBS for nuclear fractions. 5 µl of each standard and sample was incubated with 250 µl of Bradford Reagent (Sigma Aldrich) in a 96-well plate. Samples were shaken for 30 seconds on a plate shaker (IKA, Staufen, Germany) followed by incubation for 30 minutes at room temperature. Absorbance was measured at 595 nm using a spectrophotometer (Synergy HT). The concentration of protein in the samples was calculated by comparing the net absorbance values against the standard curve.

2.1.18 PPAR- γ DNA binding ELISA

PPREs consist of an extended 5'-half-site, a core DR1, and adenine as the spacing nucleotide between the two hexamers, giving the consensus sequence: 5'-AACT AGGTCA A AGGTCA-3'. PPAR interacts with the upstream extended core hexamer of the DR1, while retinoid-X-receptor (RXR) binds to the 3'half site. PPARs are unable to bind DNA as monomers and require the carboxy-terminal extension region in PPAR:RXR for the recognition of the 5'half site of the DR1 in PPREs (**Figure 2.5**).

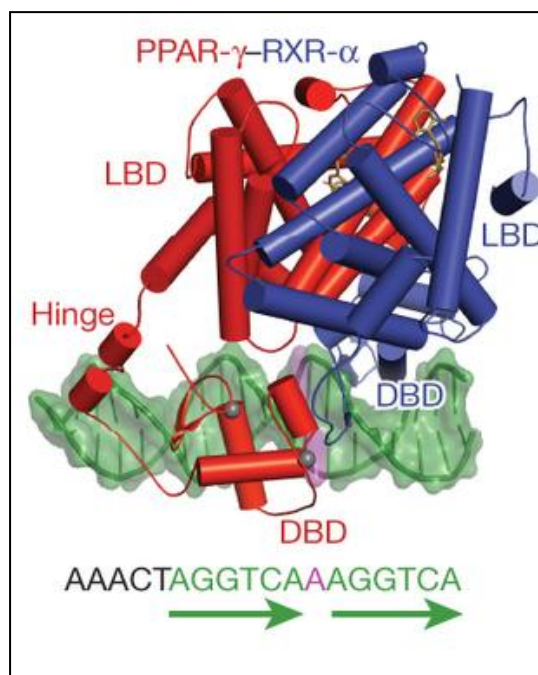


Figure 2.5 PPAR:RXR heterodimer on DR1 (*Adapted from (Chandra et al., 2013).* LBD, ligand binding domain. DBD, DNA binding domain.

Transcription factor activation (PPAR- γ binding to PPRE) was measured using a TransAM[®] DNA-binding ELISA (Active Motif). The TransAM kit consists of a 96-well plate to which oligonucleotides containing PPRE are immobilised. PPAR- γ present in nuclear extracts binds to the immobilised PPRE on the plate and is detected using an antibody directed against PPAR- γ . The addition of a secondary (detection) antibody, conjugated to HRP, produces a colorimetric readout directly proportional to the amount of PPAR- γ bound to PPRE.

Complete binding buffer (40 μ l) was added to each well of a 96-well plate. 10 μ g nuclear extract diluted in complete lysis buffer was added to each sample well. To blank wells 10 μ l complete lysis buffer was added. The plate was sealed with an adhesive cover and incubated at room temperature for 1 hour at 100 rpm with mild agitation on a rocking platform. Following incubation, wells were washed three times with wash buffer and wells were emptied by flicking the plate over the sink, then gently inverting the plate three times on a paper towel. PPAR- γ antibody was diluted in antibody binding buffer and 100 μ l was added to all wells being used. The plate was covered and incubated for 1 hour at room temperature without agitation. After incubation the plate was washed three times as described previously. Anti-mouse HRP-conjugated antibody

diluted in antibody binding buffer was added to all wells. The plate was covered and incubated for 1 hour at room temperature without agitation. After incubation the plate was washed four times as described previously. 100 μ l developing solution was added to each well and the plate was incubated for 5-10 minutes at room temperature protected from the light. The plate was observed until a medium to dark blue colour developed. 100 μ l stop solution was added immediately, which stopped the reaction, causing a colour change from blue to yellow. Absorbance was read in a microplate reader (Synergy HT; Biotek) within 5 minutes at 450 nm with a reference wavelength of 655 nm.

2.2 Human study

2.2.1 Equipment

Table 2.11 List of equipment

Item	Supplier
Jouan CR 412 centrifuge for blood handling	Thermo Scientific, Loughborough, UK
TBF 300MA bioelectrical impedance analyser	Tanita UK Ltd, Middlesex, UK
Weighing scale	Seca, Canterbury, UK
Stadiometer	Seca, Canterbury, UK
Skinfold calliper	Holtain, Crymych, UK
Tape measure	Seca, Canterbury, UK

2.2.2 Consumables

Table 2.12 Venous blood collection tubes

Vacutainer tubes	Supplier
5 ml gold tubes with clot activator and gel for serum separation	Becton Dickinson; cat no. 367954
4 ml EDTA lavender tubes	Becton Dickinson; cat no. 367839
2 ml fluoride/oxalate grey tubes	Becton Dickinson; cat no. 368920

2.2.3 Supplements

Table 2.13 CLA and placebo supplements

Item	Supplier
1 g CLA-rich soft gel capsules (Clarinol [®] A80)	Stepan Lipid Nutrition, Koog aan de Zaan, Netherlands.
1 g HOSF soft gel capsules	
CLA, conjugated linoleic acid. HOSF, high oleic sunflower oil	

2.2.4 Anthropometry

Weight and height were measured using a digital column scale and stadiometer (Seca, Birmingham, UK) to the nearest 0.1 kg and 0.1 cm, respectively. WC was measured to the nearest 0.1 cm and in the standing position with arms placed on opposite shoulders, and weight evenly distributed on each leg. The measurement was taken as the midpoint between the lowest rib and the right ilium at the midaxillary line (World Health Organisation, 2008). Where possible, WC measurements were taken with patient's waist uncovered or without heavy outer garments where this was not possible. Readings were taken at the end of a normal exhalation. Skinfolts were measured in triplicate to

the nearest 0.1 mm using a Holtain calliper (Crymych, UK) with the patient in standing position with arms hanging loosely by the side and weight evenly distributed on both legs. Skinfolts consisted of a double thickness of skin measured at four sites: triceps, biceps, suprailiac and subscapular. The triceps skinfold was measured on the right upper arm mid-point mark on the posterior surface. Skinfolts at the subscapular site were measured at the inferior angle of the right scapula. Suprailiac skinfolts were measured at the high point of the right iliac crest on the midaxillary line. The average of each skinfold was calculated and as the values obtained with the calliper are not normally distributed it was necessary to log-transform the data. The total combined skinfold thickness was calculated and the resulting values used in body density equations (**Appendix 10**) to estimate percentage body fat (Durnin and Womersley, 1974). Body fat was also assessed using the Tanita TBF 300MA according to the manufacturer's instructions with 0.5 kg allowance for clothing.

2.2.5 Dietary assessment

The EPIC-Norfolk food frequency questionnaire (FFQ) (**Appendix 11**) was used in this study to assess participants' habitual food intake. Participants were asked to complete the FFQ at baseline and week 12 to establish baseline intake and to determine whether this changed after the 12 week intervention. The FFQ is a 9-page document consisting of two parts: the first and main part asks the participant to indicate their usual consumption of 130 foods from categories ranging from "never or less than once/month" to "6 times per day". Part two consists of an additional set of questions to gather supplementary information on cooking methods and breakfast cereals, and to gain more information about milk, total fat and fatty acid intake. Data obtained from each FFQ were analysed using programs created in Microsoft Access and Excel for use in previous studies carried out at King's College London. These programs were originally set up to determine dietary intake per day for each participant for the macronutrients. Average portion sizes were derived based on those used originally in the EPIC questionnaire, values in "Food Portion Sizes" (Mills and Sejal, 2005) and "A Photographic Atlas of Food Portion Sizes" (Nelson et al., 1997).

Retrospective assessment of dietary intake was carried out by the researcher using the triple pass 24-hour recall method (Nelson et al., 2008); **Appendix 12**) at baseline and week 12 to assess participants' food intake during the previous 24 hours. The 24-hour recall was undertaken in chronological order of consumption, following a pattern of a

free and uninterrupted recall of intake, followed by detailed and specific questions about intake and quantities consumed. The recall concluded with a review of the information previously recalled, allowing for the addition of any items not remembered up to that point. Data obtained from each 24-hour recall were analysed using Microdiet™ software (Downlee Systems Ltd, High Peak, UK).

2.2.6 Physical activity

A self-administered, short version of the International Physical Activity Questionnaire (International Physical Activity Questionnaire, 2001); **Appendix 13**) was used to obtain an estimate of participants' physical activity levels (PALs) over the previous 7 days. Scores for physical activity ranging from low to moderate and high were determined for each participant based on number of days and duration of vigorous and moderate activity, and walking and are reported as metabolic equivalent (MET) minutes per week (International Physical Activity Questionnaire, 2005).

2.2.7 Blood analytic methods

Unless patient results were available within the previous 3 months, venous blood samples for the analysis of FG, TG, TC, HDL-C, LDL-C, liver function, CD4 count, HIV viral load, oxidative stress, CLA and adipocytokines were collected in evacuated tubes with the minimal compression necessary to display the vein. All blood samples were drawn after a minimum 12-hour overnight fast. Blood for glucose was collected into 4 ml fluoride oxalate vacutainers. Blood for TG, lipids and liver function was collected into 5 ml serum tubes containing a clot activator and gel for separation. Blood for CD4 count, HIV viral load, oxidative stress, CLA and adipocytokines was collected into 4 ml EDTA tubes. Blood samples used for the analysis of glucose, lipids, liver function, CD4 count and HIV viral load were sent immediately after draw to the Blood Sciences and Virology Laboratories at St Thomas' Hospital and blood for the measurement of adipocytokines was sent to the Department of Clinical Biochemistry, King's College Hospital. The author was not involved in analysing these variables. One vacutainer to be used for the analysis of CLA and oxidative stress was retained by the investigator, centrifuged at 2,000 rpm for 10 minutes at 4 °C and the plasma removed and stored at -80°C until analysis.

2.2.8 Determination of biochemical parameters

Determination of plasma TG, TC, HDL-C, LDL-C, glucose and liver function was conducted by the Blood Sciences Laboratory and CD4 count and HIV viral load conducted at the Virology Laboratory at St Thomas' Hospital. Analysis of plasma adipocytokines concentrations were conducted by the Department of Clinical Biochemistry at King's College Hospital. For detailed methodology refer to **Appendices 14-22**.

2.2.8.1 Lipids

TC was determined using an automated enzymatic method on the Roche c8000 cobas c system. Briefly, cholesterol esters were cleaved by the action of cholesterol esterase to yield free cholesterol and fatty acids. The free cholesterol was oxidised to cholesten-3-one and hydrogen peroxide by cholesterol oxidase. Peroxidase catalysed the reaction of hydrogen peroxide with 4-aminoantipyrine and phenol to produce a red coloured quinoneimine product. Colour intensity was directly proportional to concentration of cholesterol and was measured spectrophotometrically at 520 nm.

HDL-C was measured using an automated enzymatic colorimetric assay in which polyethylene glycol was used to modify cholesterol esterase and cholesterol oxidase enzymes. Cholesterol esters were broken down quantitatively into free cholesterol and fatty acids by cholesterol esterase. In the presence of oxygen cholesterol was oxidised by cholesterol oxidase to cholestenone and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide reacted with 4-amino-antipyrine to form a purple-blue dye. Colour intensity, which was directly proportional to concentration of HDL-C, was measured at 600 nm.

TG was measured using an automated enzymatic colorimetric assay. TG was hydrolyzed to glycerol and free fatty acids by lipase. Three enzymatic steps using glycerol kinase, glycerophosphate oxidase, and horseradish peroxidase caused oxidative coupling of 3,5-dichloro-2-hydroxybenzenesulfonic acid with 4-aminoantipyrine to form a red quinoneimine dye. The change in absorbance (520 nm) was directly proportional to the concentration of TG in the sample. LDL-C was calculated using the Friedwald formula if fasting plasma TG concentrations were < 4.49 mmol/l. The formula used was: $LDL-C = TC - HDL-C - (TAG/2.2)$.

2.2.8.2 Glucose

Glucose was determined using the enzymatic hexokinase/ glucose-6-phosphate dehydrogenase method. Glucose was oxidised by glucose oxidase to gluconolactone in the presence of atmospheric oxygen. Hydrogen peroxide produced as a result, oxidised 4-aminophenazone and phenol to 4-benzoquinone-monoimino phenazone in the presence of peroxidase. The colour intensity of the red dye, which is directly proportional to the glucose concentration, was measured spectrophotometrically at 340 nm.

2.2.8.3 Liver function

Albumin was determined by binding with bromocresol green (BCG) at a pH of 4.1 to form an albumin-BCG complex. The colour intensity of the blue-green colour was directly proportional to the albumin concentration and was measured spectrophotometrically at 570 nm. ALT catalyses the transamination of L-alanine and 2-oxoglutarate to pyruvate and L-glutamate. Pyruvate is then reduced to lactate in the presence of lactate dehydrogenase with the concurrent oxidation of NADH to NAD. The rate of the NAD oxidation is directly proportional to the catalytic ALT activity, which was determined by measuring the decrease in absorbance at 340 nm. For ALP, p-nitrophenyl phosphate is cleaved by phosphatases into phosphate and p-nitrophenol in the presence of magnesium and zinc ions. The p-nitrophenol released is directly proportional to the catalytic ALP activity and was determined by measuring the increase in absorbance at 480 nm. Total bilirubin in the presence of caffeine, benzoate, and acetate is coupled with a diazonium ion in a strongly acidic medium. The intensity of the colour of the azobilirubin produced is proportional to the total bilirubin concentration and was measured spectrophotometrically at 520 nm.

2.2.8.4 CD4 count and HIV viral load

Samples were prepared on the automated FP1000 system (Beckman Coulter, High Wycombe, UK) and CD4 T cell count was subsequently measured by flow cytometry using the Cytomics FC 500 (Beckman Coulter). Absolute CD4 count was determined based on the CD4 cell percentage and the total white blood cell count. HIV viral load was defined as the number of copies of HIV-1 RNA per ml of plasma and was determined by RT-PCR using the COBAS[®] AmpliPrep/COBAS[®] Taqman[®] HIV-1 Test v2.0 (Roche Diagnostics, Mannheim, Germany).

2.2.8.5 Adipocytokines

Total plasma adiponectin was determined using a Quantikine Adiponectin ELISA kit (R & D Systems). This sandwich ELISA technique consisted of a monoclonal antibody specific for adiponectin pre-coated onto a microplate. Samples were added to the wells and any adiponectin present in the sample bound to the monoclonal antibody. The addition of a substrate solution induced a colour change proportional to the amount of bound adiponectin. The colour change was measured spectrophotometrically.

Plasma cytokine concentrations (IL-2, IL-4, IL-6, IL-8, IL-10, vascular endothelial growth factor (VEGF), IFN- γ , TNF- α , IL-1 α , IL-1 β , monocyte chemoattractant protein-1 (MCP-1), epidermal growth factor (EGF)) were determined using the Evidence Investigator cytokine and growth factor array cytokine chip array kit (Randox Laboratories, Crumlin, UK), which performed simultaneous quantitative detection of adipocytokines. The Randox Biochip consisted of a biochip containing an array of discrete test regions of immobilised antibodies specific to the cytokines of interest and employed a sandwich chemiluminescent immunoassay for the cytokine array. The antibody labelled with HRP bound to the sample according to the concentration of cytokine in the sample and thus an increase in cytokine concentration led to an increase in the chemiluminescent signal emitted, which was detected using digital imaging technology.

The following tests were performed by the investigator. Determination of plasma CLA levels was conducted by the author at the Diabetes and Nutritional Sciences Division, King's College London under the guidance of Mr Robert Grey.

2.2.8.6 Determination of plasma CLA

Gas liquid chromatography (GLC) was used to quantitatively analyse fatty acid methyl esters (FAMES) derived from fatty acids in plasma samples. The basic processes involved in GLC analysis of FAMES is illustrated below in **Figure 2.6**.

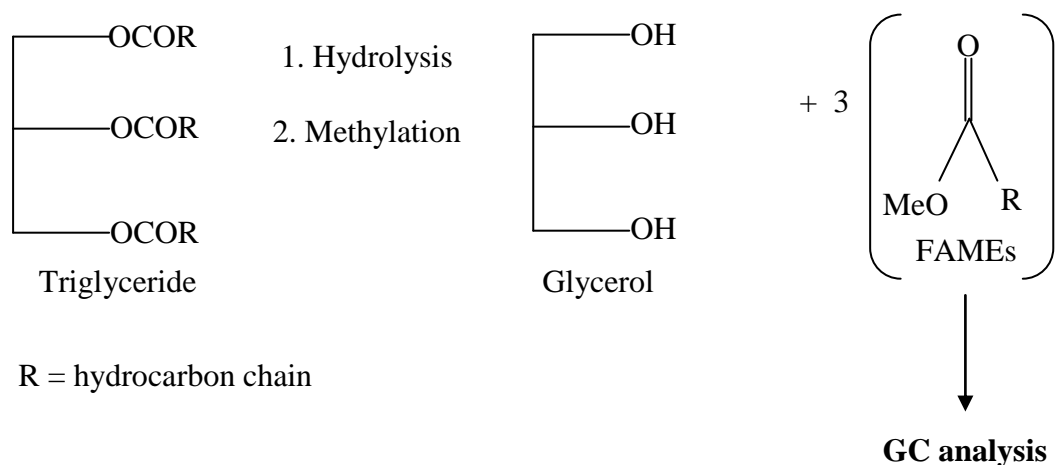


Figure 2.6 GLC analysis of fatty acid methyl esters (*Adapted from (Yung, 2013).*

GLC is used for separating components of volatile compounds based on their boiling points, as well as differences in their partition coefficients in a gaseous moving phase and a liquid stationary phase. An inert solid material acts as a support onto which the liquid stationary phase is adsorbed. The initial step in GLC analysis involves the esterification of fatty acids to FAMES. Fatty acids are bound to glycerol and circulate in the blood as TG. Therefore, these TG must be broken down to release the FFA, which are then esterified to volatile methyl esters. Analysing fatty acids as FAMES reduces their polarity making them easier to analyse (Sigma Aldrich, 2014b). Secondly, neutralising the polar carboxyl functional groups allows for the detection of small differences exhibited by unsaturated fatty acids based on their boiling points and cis and trans configuration (Sigma Aldrich, 2014b).

Esterification involves condensation of the carboxyl group of the fatty acid and the hydroxyl group of the alcohol. The addition of a catalyst allows for protonation of an oxygen atom of the carboxyl group. This makes the acid more reactive and the resulting combination with alcohol produces an ester. Use of methanol at this stage results in the formation of methyl esters (Sigma Aldrich, 2014b). The GLC output consists of a series of peaks. The first peak is the solvent peak and after this, the FAMES appear in order of chain length i.e. C10:0 before C12:0 before C14:0, etc. For a given chain length, saturated fatty acid methyl esters appear before monounsaturated before polyunsaturated.

Lipid extracts from the same subjects were treated at the same time. FAMES were created by transesterification as previously described (Lepage and Roy, 1986). 100 μ l plasma was pipetted into a Teflon lined screw cap tube. 10 ml acetyl chloride was added drop-wise into 100 ml internal standard mixture containing 20 ml Toluene and 80 ml methanol with C15:0 at 1 mg/ml. 2.2 ml of this acidic methanol solution was added to the tube. The tube was sealed and incubated overnight at 40 °C to effect transesterification. Samples were cooled and 5 ml of 6% potassium/sodium carbonate was added to neutralise excess acid. The upper organic phase of the sample was collected and transferred using a Pasteur pipette to a GLC vial. 1 μ l of each sample was injected into Agilent Technologies 6890 gas chromatograph (Agilent Technologies UK Limited) fitted with a 25 m length x 0.22 mm i.d. x 0.25 μ m film thickness Supelco SAC-5 capillary column (Sigma Aldrich) and equipped with a flame ionisation detector set at 250°C. The injection was performed in split mode (50:1) with the inlet maintained at 240°C. Temperature programming was employed to optimise the separation. This was achieved by holding the initial column temperature at 160°C for 4 min, followed by ramping at 10°C/ min to 200°C and holding at this temperature for 10 min to ensure complete elution. The identities of the separated fatty acids were established by matching their retention times with those present in authentic reference standards run under identical conditions. Proportions of fatty acids as weight % were quantified by expressing the areas under the chromatographic peaks as a % of the total integrated area. CLA isomers were identified by reference to CLA analytical standards (Sigma Aldrich).

2.2.8.7 Oxidative stress

Free-radicals are produced under conditions of oxidative stress, which induce peroxidation of PUFA in the phospholipid bilayer leading to the formation of bioactive aldehydes (Catalá, 2013). Malondialdehyde (MDA) is an example of one of these aldehydes and is an end-product of PUFA peroxidation. MDA reacts with thiobarbituric acid reactive substances (TBARS) under high temperature and acidic conditions to form MDA-TBARS adducts, which can be measured colorimetrically as a marker of lipid peroxidation (Pizzimenti et al., 2013). Measurement of TBARS formation has been used previously to determine oxidative stress in HIV-positive individuals in response to acute exercise regimes (Deresz et al., 2010). Lipid peroxidation was measured in the current study as a marker of safety, as studies have shown that CLA supplementation can cause an increase in markers of oxidative stress (Turpeinen et al., 2008).

Frozen samples were thawed on ice for 30 minutes. 100 µl of each sample was used in a TBARS assay kit (Cayman Chemicals, Cambridge Bioscience, Cambridge, UK) to determine lipid peroxidation as an indicator of oxidative stress. Standards were prepared from a stock solution of 125 µM MDA by serial dilution in the range 0- 50 µM. 100 µl sample or standard was added to a labelled vial, to which 100 µl SDS solution was added. 4 ml of a colour reagent consisting of thiobarbituric acid (TBA) acetic acid solution and TBA sodium hydroxide was then added to the vial which was boiled for 1 hour. Vials were removed immediately and placed in ice water for 10 minutes. Vials were centrifuged at 2,750 rpm for 4 minutes. 150 µl of each sample was then loaded onto a colorimetric plate in duplicate and the absorbance of the solution read at 540 nm in a spectrophotometer (Synergy HT). A standard curve was constructed by plotting the mean absorbance values (optical density) from the standard solutions versus the known MDA concentrations. Sample concentrations were calculated using the equation of the standard curve.

Chapter 3

The effect of antiretroviral drugs on 3T3-L1 adipocytes

3.1 Introduction

Abnormalities of body fat distribution are a characteristic feature of HALS (Galescu et al., 2013). The extent to which different ARVs induce these abnormalities varies greatly between and within the drug classes. Although Carr *et al* (1998a) originally attributed the abnormalities observed in HALS to PIs, other researchers showed that these abnormalities also occurred in those not receiving PIs (Lo et al., 1998). PIs are known to cause central fat accumulation and IR (Carr et al., 1998a), while NRTI have been associated with the development of LA of the face and extremities (Nolan, 2003). In contrast, NtRTI-based regimens have been shown to be beneficial in improving LA (Ribera et al., 2013), highlighting the fact that not all ARVs are equal in their effects on adipose tissue.

During the process of adipogenesis, fibroblast-like mesenchymal precursor cells proliferate and commit to differentiation along an adipocyte lineage. Under hormonal stimulation, the resulting preadipocytes undergo mitotic clonal expansion and numerous rounds of cell division. The expression and activity of the transcription factors CEBP- β and CEBP- δ induces PPAR- γ and CEBP- α expression in the terminal stages of differentiation, which activate the transcription of numerous lipid metabolism genes including adipocyte lipid binding protein, glucose metabolism genes such as GLUT4 and secreted factors such as adiponectin, leptin and resistin (Ahmadian et al., 2013). PPAR- γ , CEBP- α and CEBP- β maintain the expression of lipid and glucose metabolism genes (Lefterova and Lazar, 2009). Lipid droplets occupy the majority of a mature adipocyte and their size is maintained by a fine balance between lipid droplet proteins such as perilipin and members of the Cide family, and lipolytic enzymes such as HSL and adipose triglyceride lipase (Suzuki et al., 2011).

Disruption of this adipogenic process has been shown to play a key role in the pathogenesis of HALS (Caron et al., 2009). ARVs have been shown to induce adipocyte dysfunction by inhibiting adipocyte differentiation and adipogenesis (Grigem et al., 2005), increasing lipolysis (Adler-Wailes et al., 2005) and increasing pro-inflammatory cytokine secretion (Johnson et al., 2004). The group of Caron *et al* (2009) suggest that ARV-induced down-regulation of PPAR- γ contributes to adipocyte dysfunction in HALS. Data on the effect of ARVs on PPAR- γ are inconsistent; some studies have shown ARVs to down-regulate PPAR- γ *in vitro* (Vernochet et al., 2005, Caso et al., 2010) and in adipose tissue biopsy samples from HALS patients (Gallego-Escuredo et

al., 2013), while other *in vitro* studies show no effect (Grigem et al., 2005, Kim, 2006, Adler-Wailes et al., 2008). PPAR- γ target genes such as adiponectin are also down-regulated by ARVs (Lagathu, 2007). The use of synthetic PPAR- γ ligand rosiglitazone has been shown to enhance PPAR- γ and target gene expression in adipose tissue of HALS patients (Sutinen et al., 2004) and to improve LA (Raboud et al., 2010), which provides further support for the role of PPAR- γ in HALS.

To date, no studies have collectively examined the effect of ARVs on PPAR- γ and its target genes involved in adipocyte differentiation, function, and inflammation. Furthermore, although it is plausible to suggest that down-regulation of PPAR- γ may be responsible for ARV-induced adipocyte dysfunction, little is known about the effects of ARVs upstream of PPAR- γ , which may account for these effects.

3.2 Hypothesis

ARVs dysregulate adipocyte function, differentiation and inflammatory profile.

3.3 Aims

The specific aims of this study were to investigate the effects of PI (RTV and IDV), NRTI (d4T and ZDV) and NtRTI (TDF) on:

- adipocyte function (TG storage)
- expression of markers of adipocyte differentiation (PPAR- γ , adiponectin, perilipin, Cidea)
- expression of markers of inflammation (IL-6, leptin, resistin)
- global gene expression

in the murine 3T3-L1 adipocyte cell line.

3.4 Methods

3.4.1 Experimental model

The 3T3-L1 murine adipocyte cell line was maintained as outlined in **Section 2.1.3**. Cells were enumerated by Trypan Blue staining and seeded at a density of 5×10^5 cells in 75 ml tissue culture flasks. Once confluent, cells were sub-cultured, enumerated and re-seeded at a density of 2.4×10^5 cells in 6-well plates and 0.6×10^5 cells in 24-well plates. Cells were grown to 80%-90% confluence in induction medium (day 1-5) and incubated for a further 48 hours to initiate growth arrest. On day 7, the medium was removed and replaced with adipogenic medium consisting of induction medium plus IBMX, dexamethasone and insulin. After two days, (day 9) this medium was removed and replaced with new medium containing insulin only in addition to the ARVs. Thereafter, the medium was changed every 2 days until cells were fully differentiated at day 14. Cells were exposed to the respective treatments for a total of five days. All experiments were conducted on mature adipocytes at day 14.

3.4.2 Antiretroviral drug treatments

Differentiated adipocytes were treated with near C_{\max} concentrations of ZDV (1 μ M), d4T (10 μ M), RTV (20 μ M), IDV (10 μ M) and TDF (1 μ M) for 5 days in 6- or 24-well plates as described in **Section 2.1.6**. Near C_{\max} concentrations were obtained from published literature (Caron et al., 2004, Viengchareun et al., 2007, Minami et al., 2011) and were deemed non-toxic by MTS cell viability assays. The vehicle for the ARVs was 0.1 % ethanol, which was received by all treated cells.

3.4.3 Oil Red O staining

Once adipocytes reached maturity (day 14), induction medium was removed and cells were washed once with PBS. Cells were formalin fixed for 1 hour. Formalin was removed, allowed to dry and the cells were washed with 60% isopropanol. Isopropanol was removed and cells were incubated in ORO working solution for 10 minutes. The ORO was removed and the cells were washed four times with distilled water. The ORO was then eluted by incubating in 100% isopropanol for 10 minutes. A volume of this solution was then transferred to a 96-well plate and the optical density of the solution was measured as described in **Section 2.1.9**.

3.4.4 Real time RT-PCR

At day 14, total RNA was extracted from adipocytes using the RNeasy Mini Kit as described in **Section 2.1.10**. Briefly, cells were lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate-containing buffer to inactivate RNases and ensure purification of intact RNA. Ethanol was added to provide appropriate binding conditions, and the sample was then applied to an RNeasy Mini spin column, where total RNA binds to the membrane and contaminants were efficiently washed away. High-quality RNA was then eluted in RNase-free water. RNA was quantified using the NanoDrop[®] spectrophotometer. cDNA fragments were generated by reverse transcription using the High Capacity RNA-to-cDNA kit as described in **Section 2.1.12**. TaqMan[®] RT-PCR was performed for PPAR- γ , adiponectin, perilipin, Cidea, leptin, resistin, IL-6 and Hprt (endogenous housekeeping control) using pre-developed assay kits and the ABI Prism 7000 sequence detection system (see **Section 2.1.13**).

3.4.5 Sandwich and multiplex ELISA

On day 14, culture medium was removed and centrifuged at 5,000 rpm for 10 minutes. Supernatants were removed from the resulting adipocyte pellets and stored at -80 °C. Secretion of adiponectin into supernatant was quantified by DuoSet ELISA (**Section 2.1.15.1**) and secretion of leptin, resistin and IL-6 were quantified by Multiplex ELISA (**Section 2.1.16**).

3.4.6 PPAR- γ consensus site binding

Nuclear activation of PPAR- γ (binding to PPRE) was investigated using a DNA binding ELISA TransAM[™] PPAR- γ Transcription Factor Assay Kit (Active Motif Europe, Rixensart, Belgium) (**Section 2.1.18**). Briefly, complete binding buffer was added to each well of a 96-well microplate to which the PPRE consensus sequence (5'-AACTAGGNCAAAGGTCA-3') was immobilised. 10 μ g nuclear extract from ARV and ethanol treated cells was diluted in lysis buffer and added to each well. The plate was incubated for one hour at room temperature with gentle agitation. Primary antibody was directed against PPAR- γ and a secondary anti-mouse IgG antibody conjugated with HRP were added to each well. Absorbance was read in a spectrophotometer at 450 nm with a reference wavelength of 655 nm.

3.4.7 Global gene expression assay

Microarray analysis was carried out on RNA extracted from adipocytes treated with control, RTV and TDF to provide support for the RT-PCR data and to identify any additional and novel pathways which might be affected by the drugs. On day 14, total RNA was extracted from adipocytes using the RNeasy Mini Kit as described in **Section 2.1.10**. RNA samples were prepared for microarray analysis using MessageAmp™ Premier RNA Amplification Kit as described in **Section 2.1.14**. RNA samples were pooled and 500 ng of each pooled sample in 5 µl was used in the reactions. The concentration of the resulting RNA was determined using the NanoDrop® spectrophotometer. Biotinylated amplified RNA (aRNA) was fragmented prior to hybridisation using the 5X Array Fragmentation Buffer supplied with the MessageAmp™ Kit. The sample was then evaluated using the Agilent®2100 Bioanalyser instrument and RNA 6000 Nano Kit loaded with 300 ng of aRNA per well. 11 µg of aRNA was hybridised onto the Affymetrix Mouse Genome 430 2.0 Array Kit (Affymetrix, UK) and was subsequently washed, stained and scanned on the Fluidics Station 400 and the GeneArray Scanner (Affymetrix, UK) according to the manufacturer's protocol. Data were analysed using the RMA algorithm and the average change in expression (ratio of treatment/ control) determined for each gene. Metacore™ pathway analysis software (GeneGo, Thomson Reuters) was used for functional analysis of differentially expressed genes and pathway analysis.

3.4.8 Statistical analysis

Statistical analyses were performed using SPSS software (version 20; IBM Corp, Armonk, New York). Data were analysed using one-way analysis of variance (ANOVA), with post-hoc analysis using Dunnett's test after demonstration of significant differences by ANOVA. Differences were considered significant if $P < 0.05$. Data are expressed as the mean \pm standard error of the mean (SEM) of four parallel observations unless otherwise stated. For microarray analysis, a two-fold change relative to control (signal log ratio ≥ 1.0 or ≤ -1.0) was selected as the threshold to define differentially expressed genes.

3.5 Results

3.5.1 Cell viability

The effect of near C_{\max} concentrations of ARVs, obtained from published literature (Caron et al., 2004, Viengchareun et al., 2007, Minami et al., 2011), was investigated prior to all experimental work. At these concentrations, viability of cells treated with ARVs was not significantly different from vehicle control cells (**Figure 3.1**). Therefore, these concentrations were determined as optimal for future experimental work.

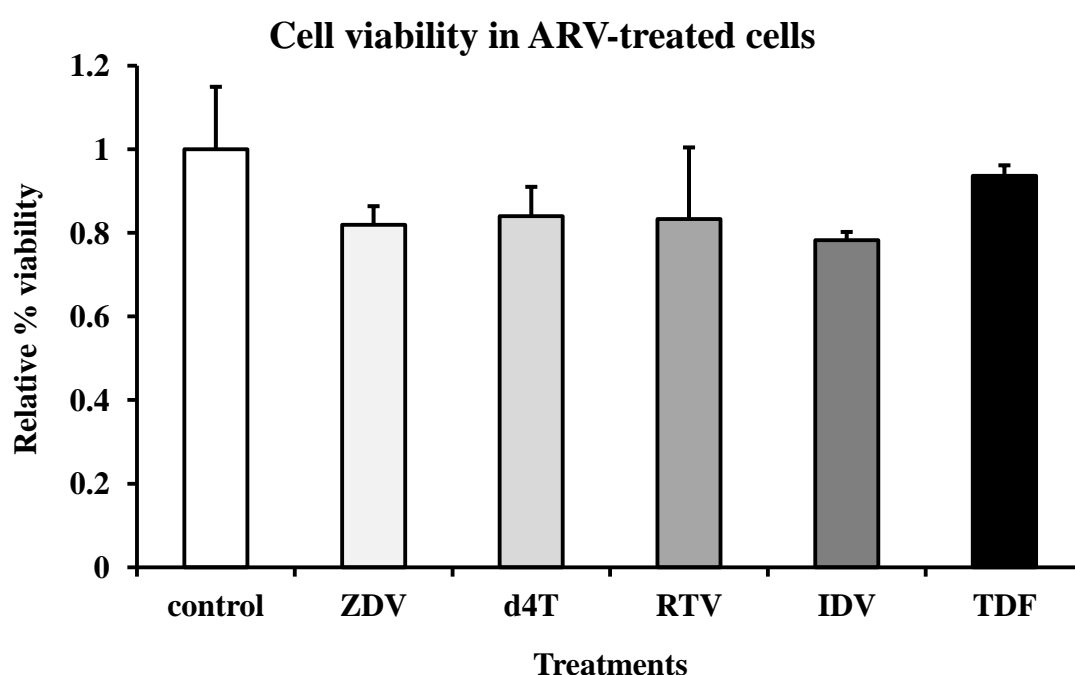


Figure 3.1 Effect of ARVs on cell viability. Adipocytes were treated with vehicle control or near C_{\max} concentrations of ZDV, d4T, RTV, IDV or TDF. An MTS assay was carried out at day 14 to determine cell viability, which was measured spectrophotometrically at 490 nm. Results represent mean \pm SEM of 4 parallel cell culture experiments. P for ANOVA = 0.258.

3.5.2 Triglyceride accumulation

To investigate the effects of each ARV on adipocyte function, TG accumulation was measured in 3T3-L1 adipocytes treated with ARVs or vehicle control (ethanol). RTV was the only ARV that significantly decreased TG accumulation (29 %) in differentiated adipocytes ($P < 0.001$; **Figure 3.2**). ORO stained images show reduced TG accumulation in RTV-treated adipocytes compared with vehicle control and other treatments (**Figure 3.3**).

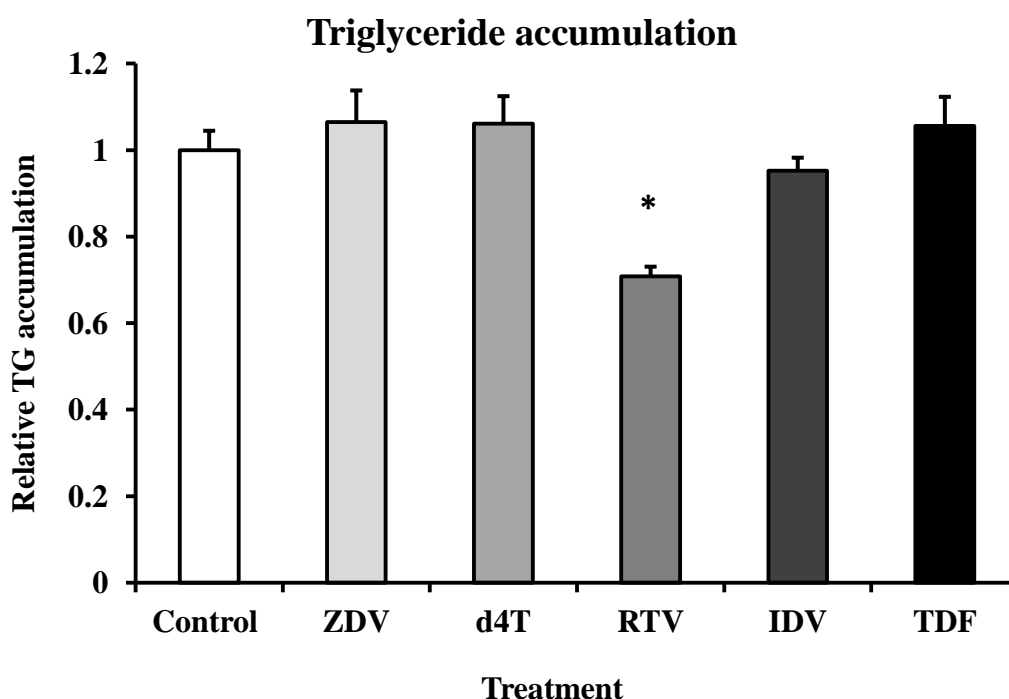
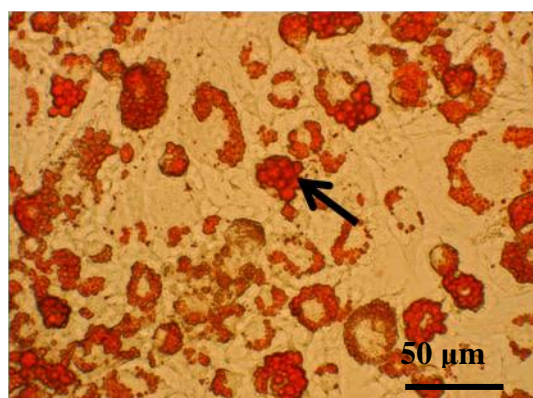
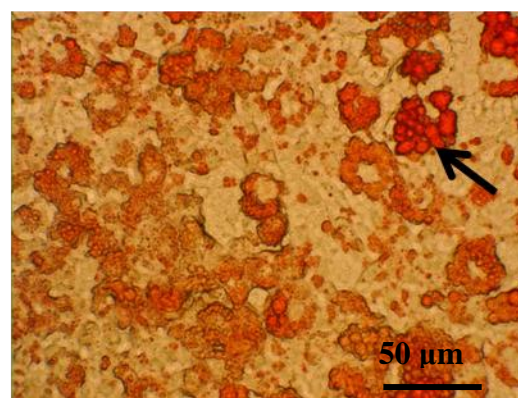


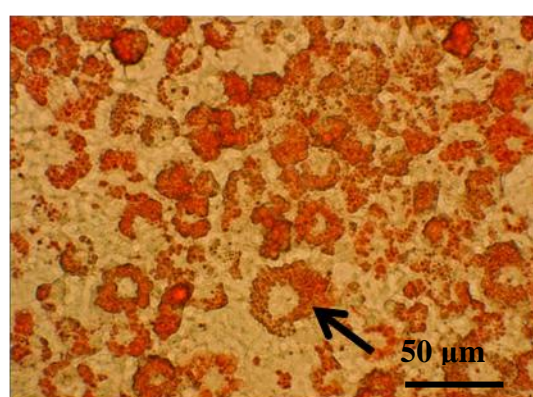
Figure 3.2 Effect of ARVs on TG accumulation. Adipocytes were treated with vehicle control or near C_{\max} concentrations of ZDV, d4T, RTV, IDV or TDF and stained at day 14 with ORO to quantify lipid content, which was measured spectrophotometrically at 540 nm. Results represent mean \pm SEM of 4 parallel cell culture experiments. *Significantly different ($P < 0.001$) from control cells.



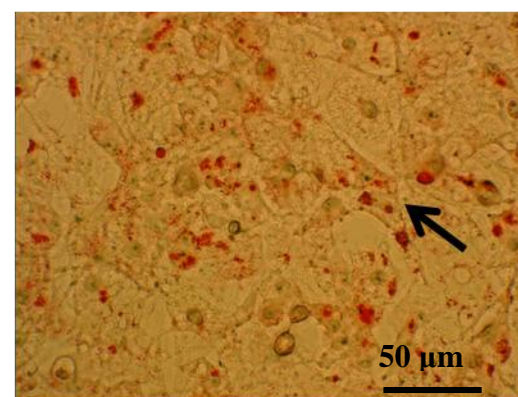
Control



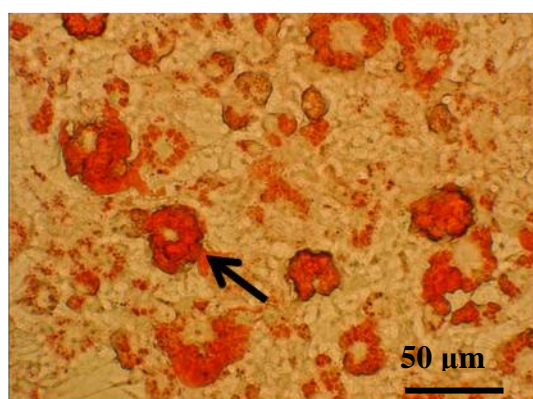
ZDV



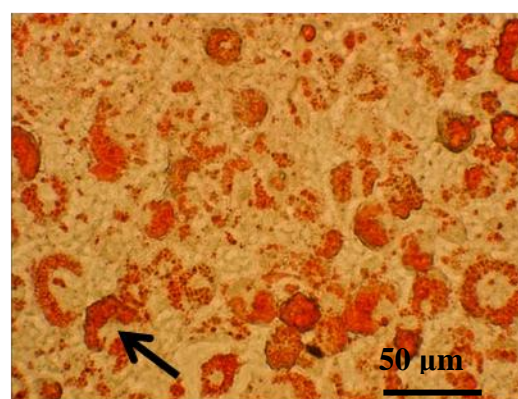
d4T



RTV



IDV



TDF

Figure 3.3 Oil Red O stained adipocytes treated with ARVs. Adipocytes were treated with vehicle control or near C_{\max} concentrations of ZDV, d4T, RTV, IDV or TDF and stained at day 14 with ORO to visualise lipid content. Black arrows indicate cells with lipid droplets and lipid vesicles (magnification is at 40x).

3.5.3 Lipid droplet protein transcription

The effect of ARVs on the expression of genes for lipid droplet-associated proteins perilipin and Cidea was investigated in 3T3-L1 adipocytes. RTV caused a significant decrease in perilipin gene expression ($P = 0.003$; **Figure 3.4**). Both RTV and IDV significantly decreased Cidea gene expression compared with control ($P < 0.001$ and $P = 0.022$, respectively; **Figure 3.5**).

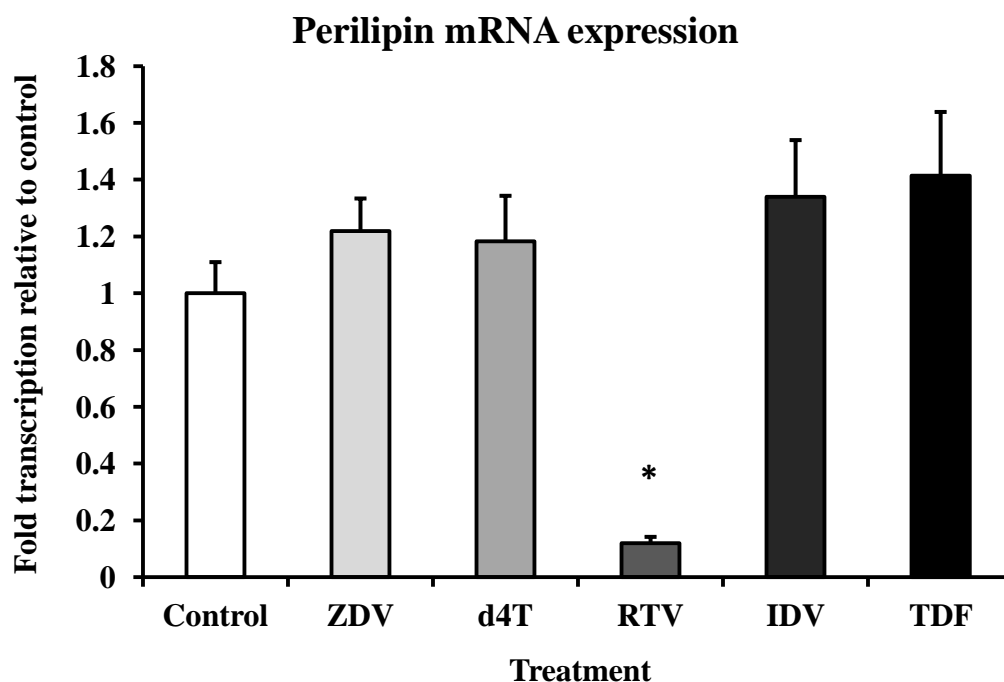


Figure 3.4 Effect of ARVs on perilipin transcription. Adipocytes were treated with vehicle control or near C_{\max} concentrations of ZDV, d4T, RTV, IDV or TDF and expression of perilipin mRNA was measured on day 14 relative to Hprt housekeeping gene by RT-PCR. Results represent mean \pm SEM of 4 parallel cell culture experiments. *Significantly different ($P 0.003$) from control cells.

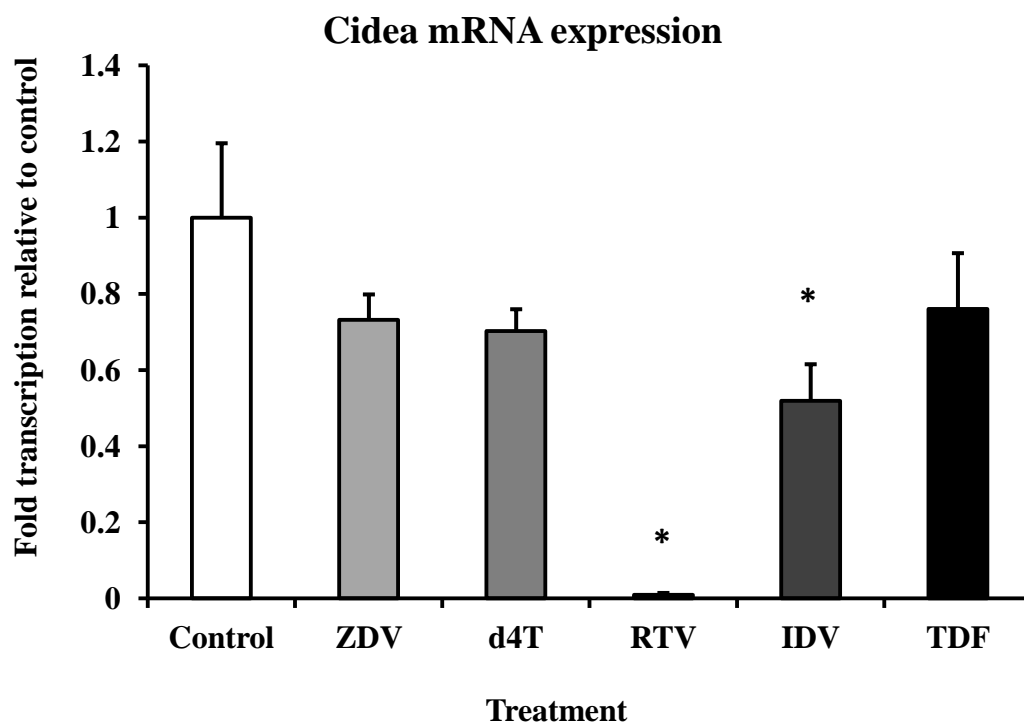


Figure 3.5 Effect of ARVs on Cidea transcription. Adipocytes were treated with vehicle control or near Cmax concentrations of ZDV, d4T, RTV, IDV or TDF and expression of Cidea mRNA was measured on day 14 relative to Hprt housekeeping gene by RT-PCR. Results represent mean \pm SEM of 4 parallel cell culture experiments. *Significantly different ($P < 0.001$ and 0.022 , respectively) from control cells.

3.5.4 PPAR- γ gene expression and nuclear activation

There was a significant reduction in PPAR- γ gene expression in RTV-treated adipocytes compared with control ($P = 0.014$; **Figure 3.6**). Nuclear PPAR- γ binding to the immobilised consensus site was also significantly reduced in cells exposed to RTV during differentiation ($P = 0.03$; **Figure 3.7**), whereas IDV and TDF significantly increased nuclear PPAR- γ binding to its consensus site ($P = 0.035$ and $P = 0.037$, respectively; **Figure 3.7**).

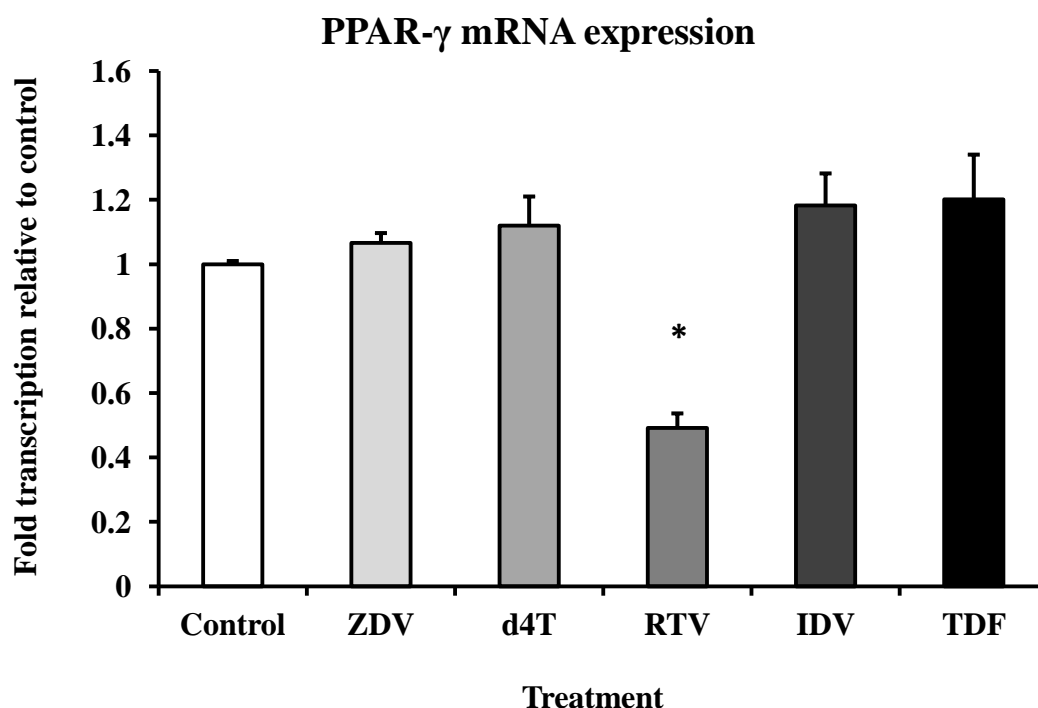


Figure 3.6 Effect of ARVs on PPAR- γ transcription. Adipocytes were treated with vehicle control or near C_{\max} concentrations of ZDV, d4T, RTV, IDV or TDF and expression of PPAR- γ mRNA was measured on day 14 relative to Hprt housekeeping gene by RT-PCR. Results represent mean \pm SEM of 4 parallel cell culture experiments. *Significantly different ($P = 0.014$) from control cells.

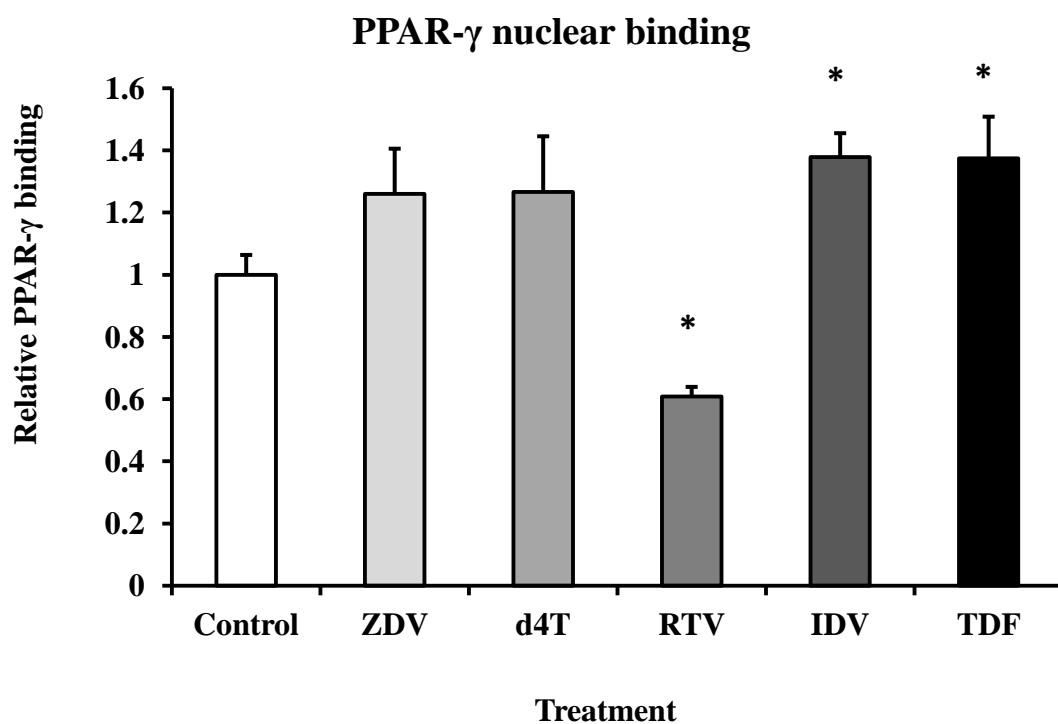


Figure 3.7 Effect of ARVs on nuclear PPAR- γ binding to PPARE. Nuclear proteins were extracted on day 14 from adipocytes treated with vehicle control or near C_{\max} concentrations of ZDV, d4T, RTV, IDV or TDF and nuclear PPAR- γ consensus site binding was measured. Results represent mean \pm SEM of 4 parallel cell culture experiments. *Significantly different ($P = 0.03$, 0.035 and 0.037 , respectively) from control cells.

3.5.5 Adiponectin gene expression and secretion

Adiponectin mRNA expression was lower by 72 % in the presence of RTV; however, this was far from significance ($P = 0.982$; **Figure 3.8**). d4T caused a significant increase in adiponectin gene expression ($P = 0.014$; **Figure 3.8**). Adiponectin protein secretion was significantly decreased by RTV, IDV and TDF ($P < 0.001$, $P = 0.004$ and $P < 0.001$, respectively; **Figure 3.9**).

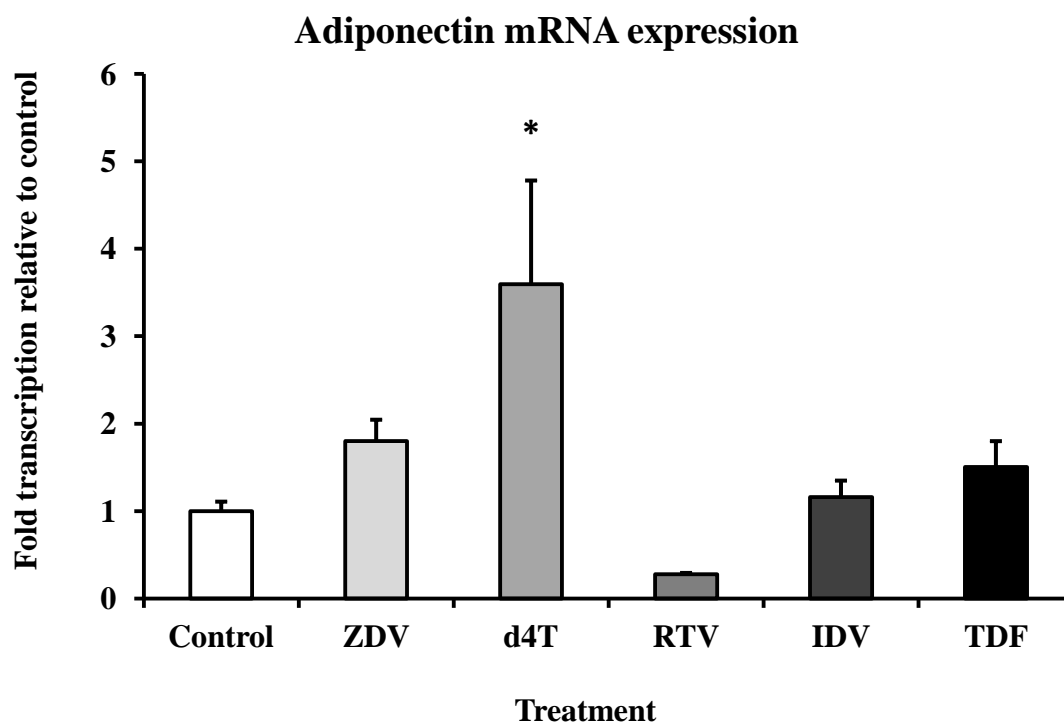


Figure 3.8 Effect of ARVs on adiponectin transcription. Adipocytes were treated with vehicle control or near C_{max} concentrations of ZDV, d4T, RTV, IDV or TDF and expression of adiponectin mRNA was measured on day 14 relative to Hprt housekeeping gene by RT-PCR. Results represent mean \pm SEM of 4 parallel cell culture experiments. *Significantly different ($P = 0.014$) from control cells.

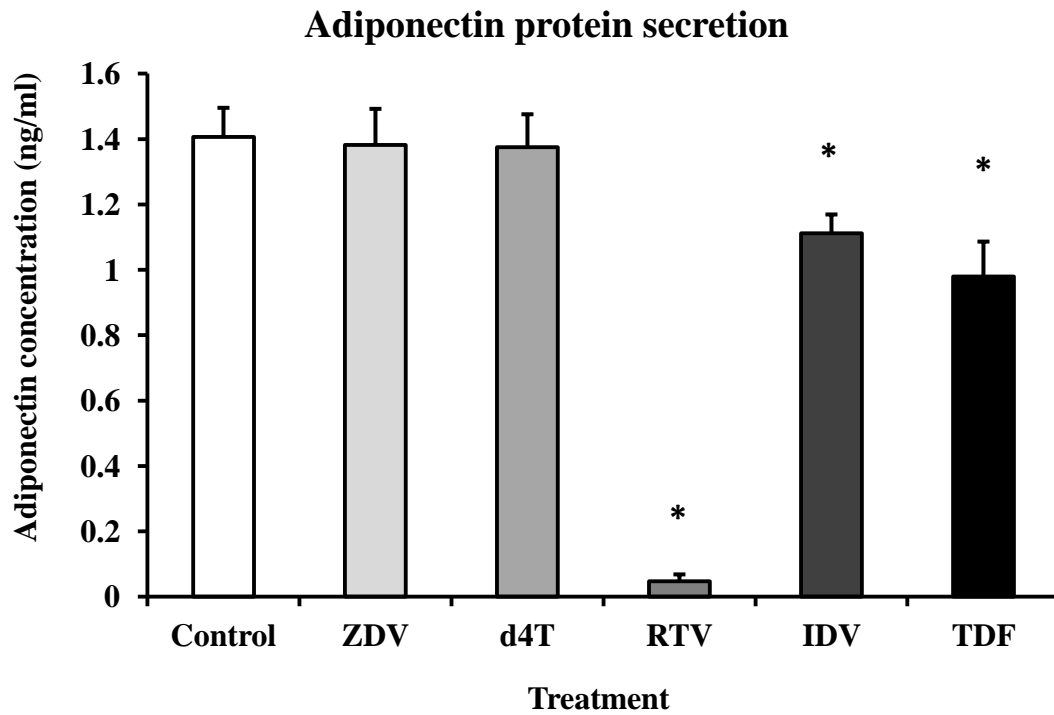


Figure 3.9 Effect of ARVs on adiponectin protein secretion. Adipocytes were treated with vehicle control or near C_{max} concentrations of ZDV, d4T, RTV, IDV or TDF. Supernatants were collected from adipocytes on day 14 and used to quantify adiponectin secretion. Results represent mean \pm SEM of 4 parallel cell culture experiments. *Significantly different ($P < 0.001$, 0.004 and < 0.001 , respectively) from control cells.

3.5.6 Leptin, resistin and interleukin-6 transcription and secretion

Expression of leptin was significantly increased in the presence of ZDV and TDF ($P = 0.021$ and $P < 0.001$, respectively; **Figure 3.10**). Leptin protein secretion was undetectable by multiplex ELISA (data not shown). TDF caused a significant increase in resistin mRNA expression ($P = 0.003$; **Figure 3.11**), while RTV significantly decreased resistin protein secretion ($P < 0.001$; **Figure 3.12**). Both IDV and TDF significantly increased IL-6 mRNA expression ($P = 0.022$ and $P < 0.001$, respectively; **Figure 3.13**). IL-6 protein secretion was undetectable by multiplex ELISA (data not shown).

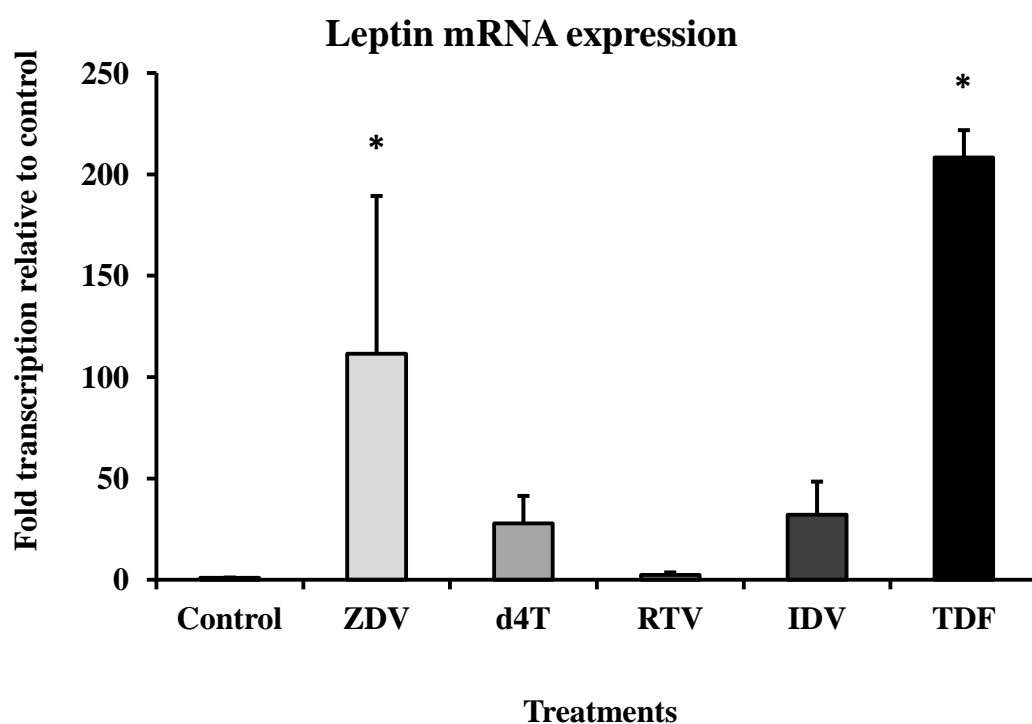


Figure 3.10 Effect of ARVs on leptin transcription. Adipocytes were treated with vehicle control or near C_{\max} concentrations of ZDV, d4T, RTV, IDV or TDF and expression of leptin mRNA was measured on day 14 relative to Hprt housekeeping gene by RT-PCR. Results represent mean \pm SEM of 4 parallel cell culture experiments. *Significantly different ($P = 0.021$ and < 0.001 , respectively) from control cells.

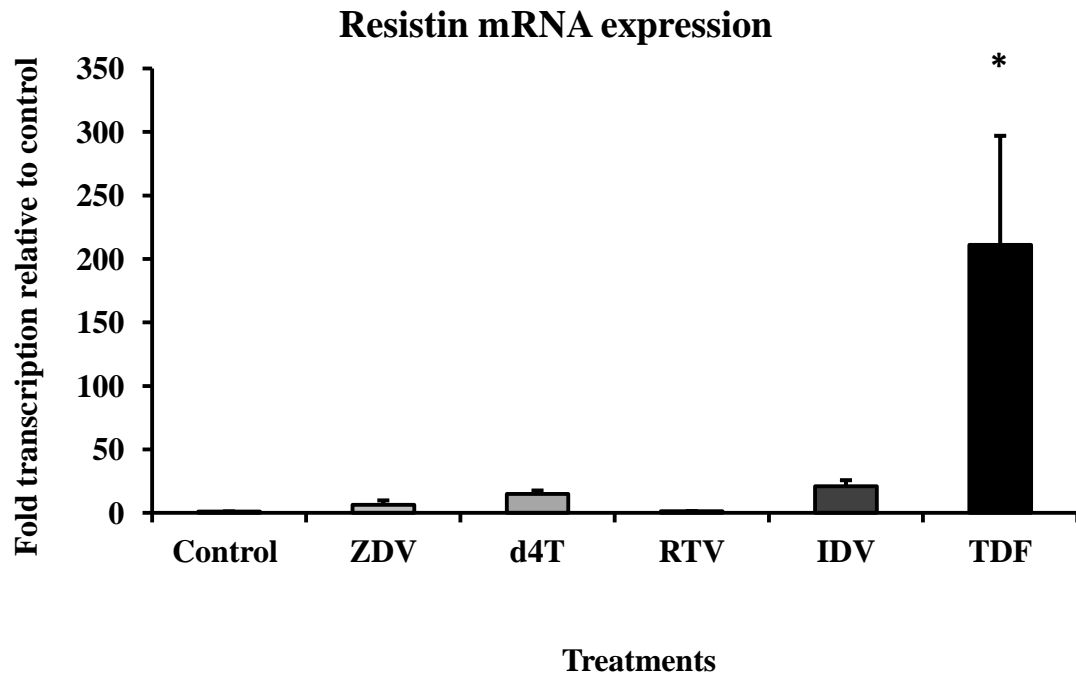


Figure 3.11 Effect of ARVs on resistin transcription. Adipocytes were treated with vehicle control or near C_{\max} concentrations of ZDV, d4T, RTV, IDV or TDF and expression of resistin mRNA was measured on day 14 relative to Hprt housekeeping gene by RT-PCR. Results represent mean \pm SEM of 4 parallel cell culture experiments. *Significantly different ($P = 0.003$) from control cells.

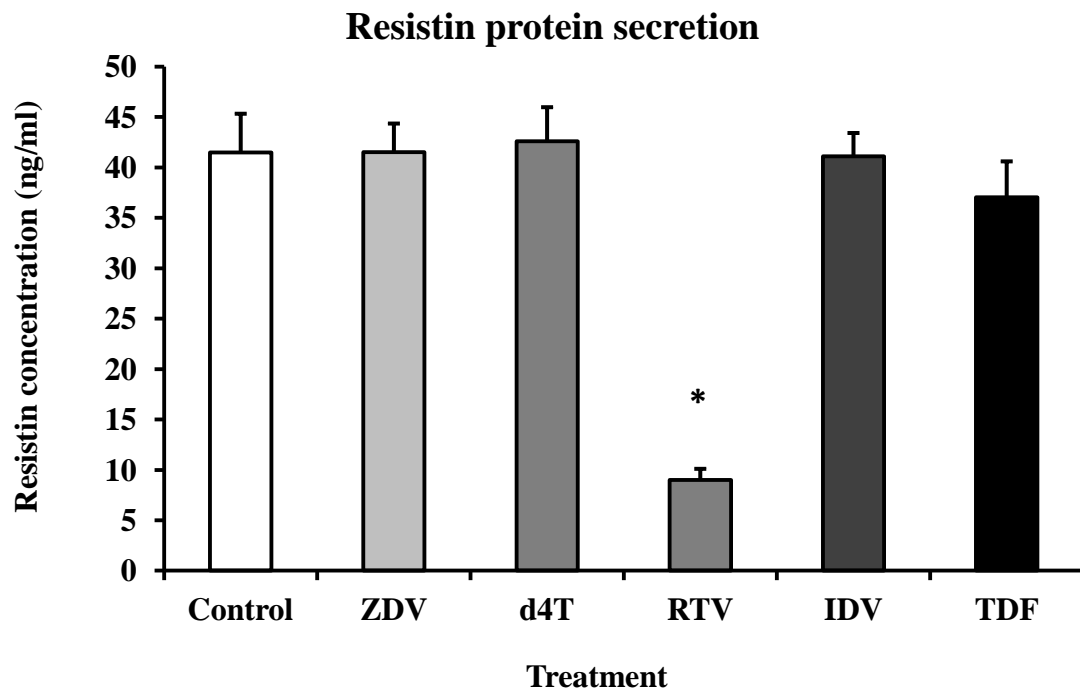


Figure 3.12 Effect of ARVs on resistin protein secretion. Adipocytes were treated with vehicle control or near C_{\max} concentrations of ZDV, d4T, RTV, IDV or TDF and resistin protein secretion was measured on day 14 using a multiplex ELISA. Results represent mean \pm SEM of 8 parallel cell culture experiments. *Significantly different ($P < 0.001$) from control cells.

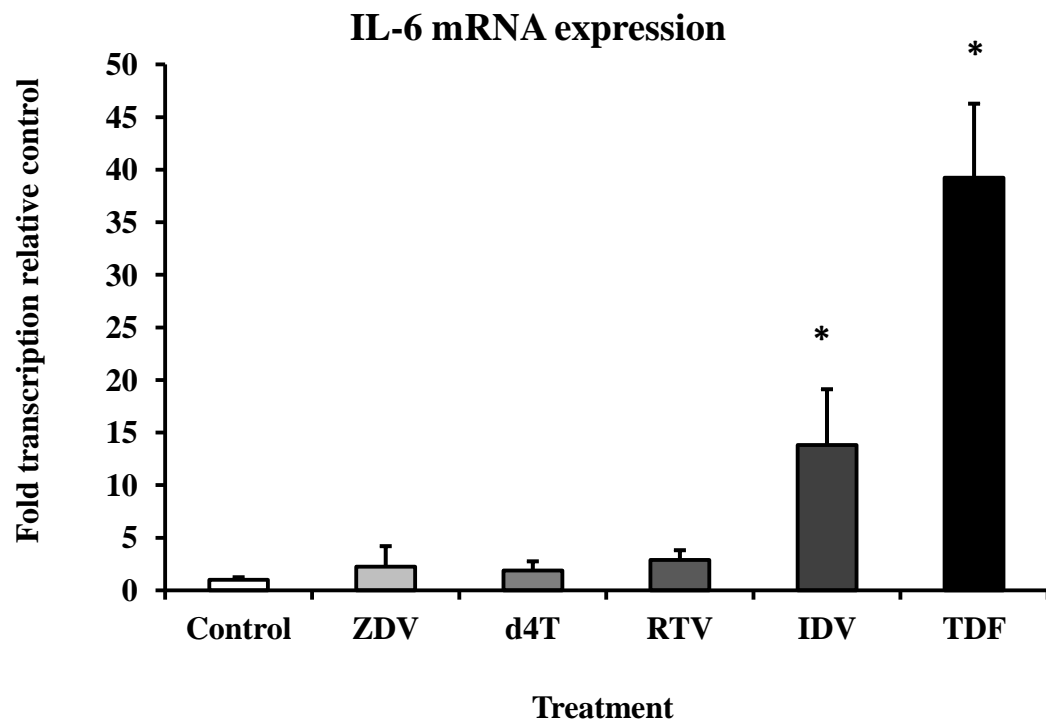


Figure 3.13 Effect of ARVs on IL-6 transcription. Adipocytes were treated with vehicle control or near C_{\max} concentrations of ZDV, d4T, RTV, IDV or TDF and expression of IL-6 mRNA was measured on day 14 relative to Hprt housekeeping gene by RT-PCR. Results represent mean \pm SEM of 4 parallel cell culture experiments. *Significantly different ($P = 0.022$ and $P < 0.001$, respectively) from control cells.

3.5.7 Global gene expression

Given the potent effect of RTV on PPAR- γ and target genes, it was decided that microarray analysis be carried out on RNA samples from RTV-treated cells. To provide a comparison, TDF, which also significantly affected a number of genes and is a newer class of ARV, was also chosen for analysis using microarray.

3.5.7.1 RTV

The expression of 1,657 genes was up-regulated greater than 2-fold and 1,379 down-regulated greater than 2-fold in RTV-treated cells compared with control. A list of key adipogenic genes of interest which were altered by RTV are shown in **Table 3.1**. Altered gene lists were imported into MetaCore™ pathway analysis software (GeneGo), which generated a list of top canonical pathway maps representing a set of signalling and metabolic maps of genes affected by RTV (**Table 3.2**). RTV had a profound effect on cell cycle pathways, with 5 of the top ten pathway maps representing changes in genes involved in cell cycle regulation. Other pathways included immune response, insulin-like growth factor (IGF) signalling and adipocyte differentiation pathways. **Figure 3.14** illustrates the pathway map for ‘Differentiation of white adipocytes’, in which 16 out of 53 genes were altered ($P = 0.007$; false discovery rate (FDR) = 0.046). Similarly, 16 out of 53 genes were altered in the ‘Insulin, IGF-1 and TNF- α in brown adipocyte differentiation’ pathway ($P = 0.007$; FDR = 0.046; **Figure 3.15**). The genes up- or down-regulated within each of these adipogenic pathways are listed in more detail in **Table 3.3**.

Table 3.1 Key genes of interest affected by RTV

Gene	Change in mRNA expression
PPAR- γ	6-fold ↓
Adiponectin	24-fold ↓
Leptin	5-fold ↓
Resistin	95-fold ↓
IL-6 was up-regulated	10-fold ↑

Table 3.1 Top 10 canonical pathways affected by RTV

	Pathway	<i>P</i> value	False Discovery Rate	Ratio*
1	Chromosome condensation in prometaphase	< 0.001	< 0.001	14/21
2	The metaphase checkpoint	< 0.001	< 0.001	18/36
3	Spindle assembly and chromosome separation	< 0.001	< 0.001	17/33
4	Role of APC in cell cycle regulation	< 0.001	0.001	16/32
5	Initiation of mitosis	< 0.001	0.001	14/25
6	Histamine H1 receptor signalling in immune response	0.001	0.005	18/48
7	IGF family signalling in colorectal cancer	0.001	0.011	19/60
8	Insulin, IGF-1 and TNF- α in brown adipocyte differentiation	0.007	0.046	16/53
9	Differentiation of white adipocytes	0.007	0.046	16/53
10	Role of Nek in cell cycle regulation	0.009	0.057	12/32

P value is based on the number of interactions of the gene in the pathway. FDR is calculated taking into account the *P* value for each map and the rank of the map, given the total number of maps in the whole Pathway Maps ontology. * Ratio of genes altered by RTV vs. total number of genes in the pathway.

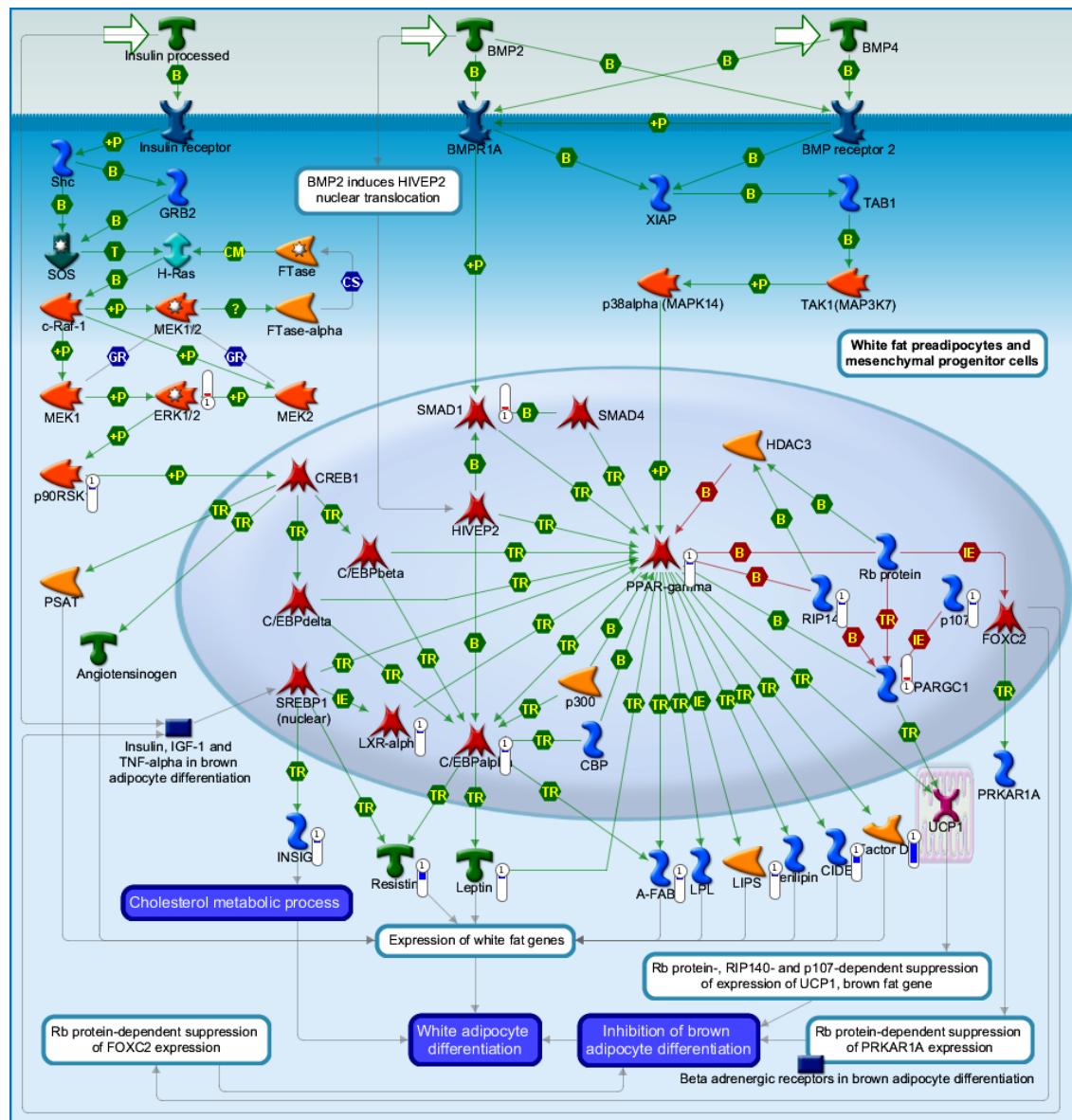


Figure 3.14 White adipocyte differentiation (GeneGo). Histograms located next to each gene indicate the direction (blue = down-regulated; red = up-regulated) and the degree of change in expression of each gene. Full figure legend can be found in **Appendix 1**.

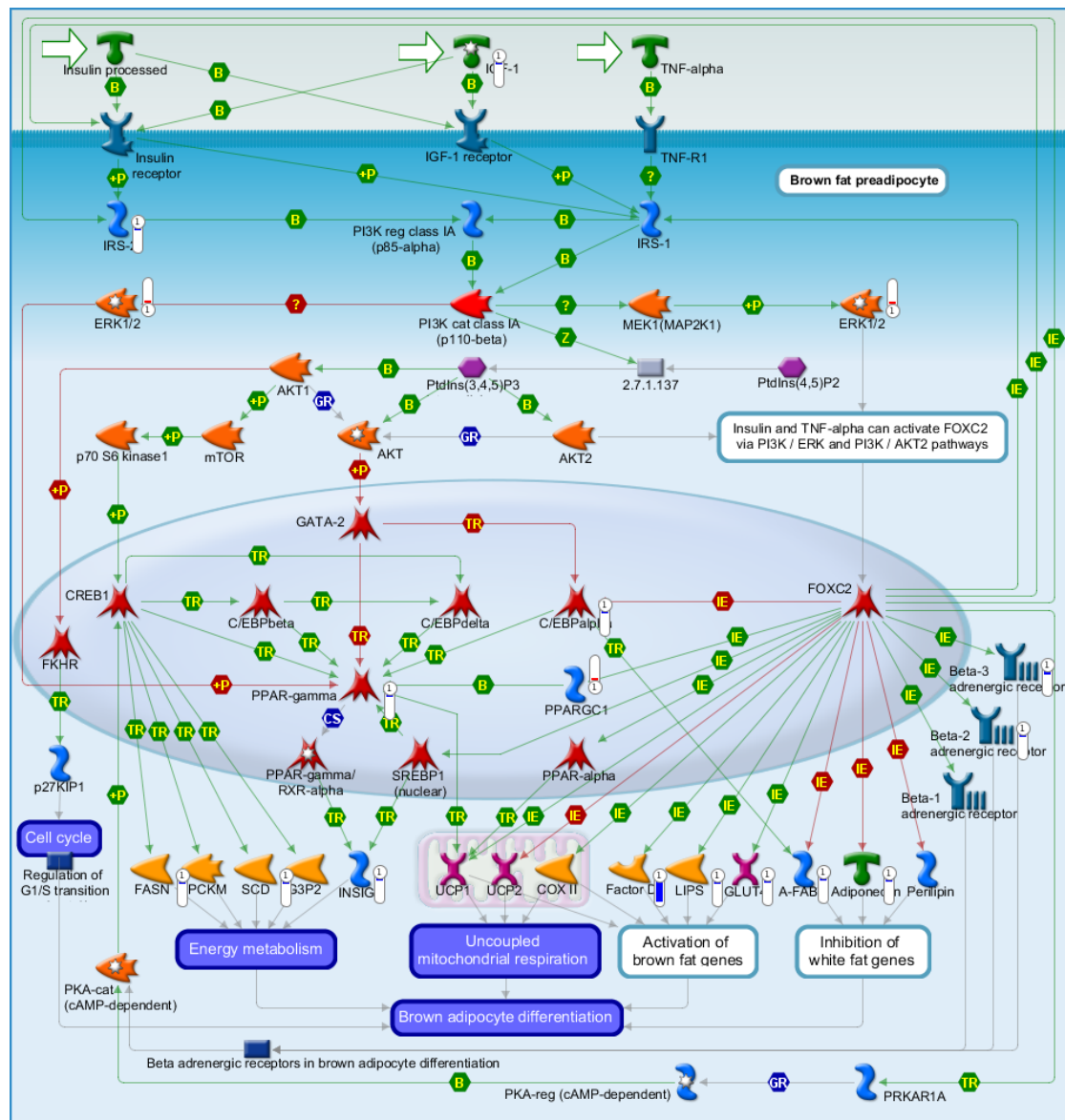


Figure 3.15 Insulin, IGF-1 and TNF- α in brown adipocyte differentiation (GeneGo). Histograms located next to each gene indicate the direction (blue = down-regulated; red = up-regulated) and the degree of change in expression of each gene. Full figure legend can be found in **Appendix 1**.

Table 3.2 Adipogenic pathways and genes altered by RTV

Pathway 8: Insulin, IGF-1 and TNF-α in brown adipocyte differentiation			
Gene name	Symbol	Description	Fold change*
Down-regulated			
Adipsin	Cfd	Stimulates glucose transport for TG accumulation in adipocytes and inhibits lipolysis	238
Adiponectin	Adipoq	Adipokine involved in fat metabolism and insulin sensitivity	24
Hormone sensitive lipase	Lips	Hydrolyses stored TG	20
Stearoyl-CoA desaturase-1	Scd1	Catalyses synthesis of unsaturated fatty acids	19.8
Glucose transporter type 4	Glut4	Insulin-dependent glucose transporter induced by PPAR- γ	13.4
CCAAT/enhancer binding protein α	Cebpa	Induces adipogenesis through PPAR- γ	11.3
Fatty acid binding protein 4	Fabp4	Carrier protein for fatty acids and other lipophilic compounds	7.5
Peroxisome proliferator-activated receptor γ	Ppar- γ	Key regulator of adipocyte differentiation	5.6
Insulin receptor substrate 2	Irs2	Up-regulates CEBP α , PPAR- γ and adipocyte differentiation	4.9
Beta 3 adrenergic receptor	Adrb3	Regulates lipolysis and thermogenesis	3
Insulin-induced gene 1	Insig1	Regulates cholesterol synthesis by blocking SREBP processing	3
Fatty acid synthase	Fasn	Catalyses fatty acid synthesis	2.8
Insulin-like growth factor 1	Igf1	Involved in adipocyte differentiation and glucose metabolism	2
Pathway 9: Differentiation of white adipocytes			
Gene name	Symbol	Description	Fold change*
Down-regulated			
Adipsin	Cfd	Stimulates glucose transport for TG accumulation in adipocytes and inhibits lipolysis	238
Resistin	Retn	Involved in IR in obesity	95
Cell death-inducing DFFA-like effector c	Cidec	Lipid droplet protein. Inhibits lipolysis in favour of lipid storage	74

Gene name	Symbol	Description	Fold change*
CCAAT/enhancer binding protein α	Cebpa	Induces adipogenesis through PPAR- γ activation	11
Fatty acid binding protein 4	Fabp4	Carrier protein for fatty acids and other lipophilic compounds	7.5
Liver X receptor α	Nr1h3	Activation enhances lipogenesis	7
Peroxisome proliferator-activated receptor γ	Ppar- γ	Key regulator of adipocyte differentiation	6
Leptin	Lep	Energy homeostasis and regulation of TG storage in adipose tissue	4.8
Ribosomal s6 kinase	Rps6kb1	Cell proliferation, survival and differentiation	3
Insulin-induced gene 1	Insig1	Regulates cholesterol synthesis by blocking SREBP processing	3
Nuclear receptor interacting protein 140	Nrip1	Blocks the expression of genes involved in energy dissipation and mitochondrial uncoupling	2.5
Retinoblastoma-like protein 1	Rbl1	Regulator of cell division	2.1
Up-regulated			
Mothers against decapentaplegic homolog 1	Smad1	Regulates adipocyte differentiation through PPAR- γ activation	3.2
Extracellular signal-regulated kinase 1/2	Erk 1 / 2	Phosphorylates nuclear receptors including PPAR- γ . Involved in cell cycle regulation	2.2
PPAR- γ coactivator 1	Ppargc1	Regulates energy metabolism genes through interaction with PPAR- γ	2.1

* fold change compared with control (ethanol)

3.5.7.2 TDF

Unlike RTV, no relevant genes were up- or down-regulated greater than 1.5- or 2-fold compared with control in TDF-treated cells and so altered gene lists could not be explored further using MetaCore™.

3.6 Discussion

In this study, ARVs had a significant effect on adipocyte function, differentiation and inflammatory cytokines. We have demonstrated the detrimental effects of a number of ARVs, RTV in particular, on TG accumulation, lipid droplet-associated proteins, PPAR- γ , adiponectin, leptin, resistin and IL-6. Furthermore, microarray analysis has identified novel effects of RTV on a number of adipogenic genes, as well as upstream regulators of PPAR- γ , which has not previously been reported.

3.6.1 Effect of ARVs on triglyceride accumulation

RTV significantly decreased TG accumulation, while the other ARVs had no significant effect. Moreover, adipocytes treated with RTV appear less well differentiated in Oil Red O stained images. This finding is in agreement with results from previous studies using the well-characterised 3T3-L1 cell line (Minami et al., 2011). A number of studies have demonstrated a detrimental effect of RTV on lipid accumulation in other cells types including human SGBS adipocytes (Kim, 2006), C3H10T1/2 stem cells (Lenhard et al., 2000) and adipocytes isolated from human subcutaneous (Lagathu, 2007) and omental adipose tissue (Jones et al., 2008). Interestingly, at concentrations of just 10 μ M, RTV induced a 50% reduction in insulin-stimulated lipogenesis in SGBS adipocytes (Grigem et al., 2005). These findings, as well as our own, exemplify the lipoatrophic effect of RTV.

3.6.2 Effect of ARVs on lipid droplet-associated proteins

An analysis of lipid droplet-associated proteins was undertaken in an attempt to investigate their role in the observed effects on TG accumulation. Consistent with the finding of reduced TG accumulation in RTV-treated cells, expression of perilipin was also reduced by RTV and Cidea expression by RTV and IDV. In contrast, microarray analysis did not find any significant alteration in perilipin or Cidea mRNA expression in response to RTV treatment. However, Cidec, another member of the Cide family, was down-regulated 74-fold by RTV in microarray analysis. Cidec regulates lipid droplet size by binding to lipid droplets and restricting lipolysis, thereby enhancing TG storage. This has been suggested to occur via interaction of Cidec with perilipin (Puri et al., 2007). Only one study has previously investigated the effect of ARV on Cidec expression and found that saquinavir significantly decreased its expression (Bociaga-Jasik et al., 2013). Our findings for perilipin and Cidea substantiate those of Adler-Wailes *et al* (2005) and (2008). Lipid droplet-associated proteins, perilipin and Cidea

act as a barrier, protecting stored TG from the lipolytic action of cellular lipases (Puri et al., 2008). RTV and other drugs from the PI family have previously been shown to decrease perilipin expression in 3T3-L1 adipocytes, which was associated with an increase in glycerol and FFA release (Adler-Wailes et al., 2005, Kovsan et al., 2007). Although lipolysis was not measured in our study, the observed reduction in TG accumulation may be due to the decreased perilipin expression as a result of RTV treatment. Furthermore, both perilipin and Cidea are PPAR- γ target genes (Dalen et al., 2004, Puri et al., 2008); therefore, down regulation of PPAR- γ in this study may have mediated the reduction in lipid droplet proteins observed in RTV-treated adipocytes.

3.6.3 Effect of ARVs on PPAR- γ

PPAR- γ plays a central role in regulating adipogenesis and lipid metabolism in mature adipocytes (Desvergne and Wahli, 1999). Binding of lipophilic ligands to PPAR- γ leads to the transcription of adipocyte genes including perilipin (Dalen et al., 2004), Cidea (Puri et al., 2008) and adiponectin (Iwaki et al., 2003). Both IDV and TDF significantly increased PPAR- γ binding to PPRE. In contrast, previous studies report decreased PPAR- γ expression in response to IDV (Caron et al., 2001, Viengchareun et al., 2007), while the effect of TDF on PPAR- γ expression has not previously been investigated *in vitro*. RTV significantly decreased PPAR- γ gene expression and binding to its PPRE. Microarray analysis confirmed these findings and showed a 6-fold decrease in PPAR- γ expression compared with control. Our results substantiate previous reports for PPAR- γ gene and protein expression *in vitro* (Dowell et al., 2000, Vernochet et al., 2005, Caso et al., 2010). However, it is worth noting that a number of studies have failed to find an effect of ARVs on PPAR- γ using near C_{\max} concentrations (Grigem et al., 2005, Kim, 2006, Adler-Wailes et al., 2008). The group of Caron *et al.* (2009) highlight a central role for PPAR- γ down-regulation in the aetiology of adipocyte dysfunction in HALS. Down-regulation of PPAR- γ has been correlated with decreased expression of adiponectin and increased expression of IL-6 (Bastard et al., 2002b, Kannisto et al., 2003, Vigouroux et al., 2003, Jan et al., 2004). PPAR- γ is essential for adipogenesis (Tontonoz and Spiegelman, 2008). Therefore, it is plausible to suggest that down-regulation of PPAR- γ leading to reduced adipogenesis may be responsible for the reduction in TG accumulation observed in RTV-treated cells in this study.

3.6.4 Effect of ARVs on adiponectin

Adiponectin is an insulin-sensitising adipokine induced during adipocyte differentiation (Scherer et al., 1995). In the current study, d4T caused an unexpected increase in adiponectin gene expression. However, data from microarray analysis show a 24-fold decrease in adiponectin mRNA expression in RTV-treated cells compared with control. When protein secretion was investigated, it was found that RTV, IDV and TDF significantly decreased adiponectin protein secretion. In contrast to RT-PCR data, previous studies have shown a reduction in adiponectin in response to d4T (Jones et al., 2005, Stankov et al., 2007). This, together with the fact that microarray and protein secretion data do not confirm this effect, may indicate that the observed effect of d4T on adiponectin expression is an anomaly. A number of other studies have demonstrated effects for PIs (RTV, amprenavir, atazanavir and abacavir) on adiponectin mRNA expression (Grigem et al., 2005, Kim, 2006, Lagathu, 2007, Adler-Wailes et al., 2008). Consistent with our findings for adiponectin protein secretion, two other research groups demonstrated a significant reduction in adiponectin gene expression in SGBS adipocytes in response to 20 μ M RTV (Grigem et al., 2005, Kim, 2006). In adipocytes derived from adult stem cells, RTV induced a significant decrease in the expression of PPAR- γ and adiponectin (Vernochet et al., 2005). A study of human adipocytes cultured in the presence of RTV has shown reduced expression, secretion and release of adiponectin (Lagathu, 2007). Furthermore, hypoadiponectinaemia is a common finding in individuals with HALS (Mynarcik et al., 2002, Addy et al., 2003, Sutinen et al., 2003, Vigouroux et al., 2003, Johnson et al., 2004, Bezante et al., 2009, Minami et al., 2009). Adiponectin plays a role in insulin sensitisation and down-regulation may contribute to IR in HIV (Kim, 2006). The precise mechanism underlying the inhibition of adiponectin is not yet clear. PPAR- γ has been suggested to play a role in the transcriptional activation of the adiponectin gene via a PPRE in the adiponectin promoter (Iwaki et al., 2003). Thus, down-regulation of adiponectin secretion in RTV-treated adipocytes in this study may be mediated by down-regulation of PPAR- γ .

3.6.5 Effect of ARVs on leptin

Leptin circulates proportional to levels of body fat and is thought to play a role in the regulation of energy intake and expenditure (Mantzoros, 2012). Leptin has been shown to be related to adiposity in HALS and to increase in response to WC (de Luis et al., 2012). In this study, the effect of ARVs on leptin was investigated using RT-PCR and microarray analysis. It was found that leptin mRNA expression was significantly

increased by ZDV and TDF. In contrast, microarray analysis showed a significant down-regulation of leptin mRNA expression in RTV-treated cells (5-fold). Decreased leptin expression in RTV-treated cells may suggest a conversion of adipocytes from cells that store TG to fatty acid oxidising cells and may be one mechanism underlying RTV-induced LA. In contrast to our RT-PCR data, Lagathu *et al* (2007) showed reduced leptin secretion in human primary adipocytes treated with ZDV. The effect of TDF on leptin mRNA has not previously been shown. Similar to our microarray data, reduced leptin secretion has also been identified in human adipocytes treated with lopinavir boosted RTV (Gallego-Escuredo et al., 2010). Hypoleptinaemia is observed in patients with HALS and leptin replacement therapy is being explored as a potential therapeutic strategy to decrease central fat mass in HALS (Mantzoros, 2012).

3.6.6 Effect of ARVs on resistin

RTV showed no effect on resistin mRNA expression in RT-PCR, but in subsequent microarray analysis, resistin expression was decreased 95-fold compared with control. For the first time, we have shown a significant increase in resistin gene expression in adipocytes treated with TDF, although no effect was observed for TDF at the protein level. In contrast to microarray data, RTV significantly decreased resistin protein secretion. Rajala *et al* (Rajala et al., 2004) also found differences in resistin mRNA and protein levels in ob/ob mice and suggest a negative feedback loop may exist whereby low circulating resistin may enhance gene expression. It is also important to note that only four replicates were performed giving a lack of power which may account for the differences observed. Resistin is an adipokine originally thought to be the link between diabetes and obesity (Steppan et al., 2001). The role of resistin in HALS is not fully understood. Ranade *et al* (2008) found a SNP in the resistin gene associated with elevated lipids, IR and body composition changes among HIV-infected individuals taking HAART. Another study found significantly greater plasma resistin levels in HALS patients compared with uninfected controls (Escote et al., 2011). However, others have found no association between serum resistin and fat redistribution, IR or metabolic profile in HIV-infected patients (Barb et al., 2005, Arama et al., 2013).

3.6.7 Effect of ARVs on IL-6

IL-6 gene expression was significantly increased by IDV and TDF in the current study. Additionally, microarray data demonstrated a 10-fold increase in IL-6 mRNA expression in response to RTV treatment. IDV has previously been shown to increase

IL-6 release from human adipocytes (Lagathu, 2007), but the effect of TDF on IL-6 expression has not been shown previously. RTV has been shown to significantly increase IL-6 expression in 3T3-F442A adipocytes (Lagathu et al., 2004) and a number of studies in SGBS (Grigem et al., 2005, Kim, 2006) and human adipocytes (Lagathu, 2007). IL-6 has been suggested to play a role in IR by blocking insulin signalling in adipocytes (Lagathu et al., 2005). Increased IL-6 has been demonstrated in SCAT from patients with HALS (Kannisto et al., 2003) and may mediate IR in these patients.

3.6.8 Global gene expression analysis

In addition to supporting RT-PCR results, pathway analysis carried out on microarray data allowed for the investigation of potential mechanisms underlying the effects of RTV on PPAR- γ and target genes. In our experiment, extracellular-signal-regulated kinase 1/2 (ERK1/2) expression was up-regulated by RTV. ERK1/2 phosphorylation of PPAR- γ results in decreased transcription and protein expression of the transcription factor, as well as a reduction in its ability to promote adipogenesis (Hu et al., 1996). Previously, PI (IDV and nelfinavir) have been shown to decrease phosphorylation of ERK1/2 in 3T3-L1 adipocytes and myocytes, which altered the activation of insulin signalling (Ben-Romano et al., 2004, Hong-Brown et al., 2004). However, the effect of ARVs on ERK1/2 in relation to PPAR- γ has not been investigated. It could be suggested that RTV-induced up-regulation of ERK1/2 in our experiment had a direct effect on PPAR- γ phosphorylation, expression and adipogenesis.

The negative effect of RTV on liver-X-receptor alpha (LXR- α) has not previously been shown and is a novel finding of this study. During adipocyte differentiation, insulin induces the expression of LXR- α , which enhances the expression of SREBP1, resulting in transcriptional activation of PPAR- γ (Seo et al., 2004). Down-regulation of LXR- α in this experiment may provide another possible mechanism for the observed down-regulation of PPAR- γ by RTV in our experiment.

Nuclear receptor interacting protein 140 (NRIP1) and retinoblastoma-like 1 (RBL1) have been shown to suppress PPAR- γ transcriptional activity (Fajas et al., 2002). In this study, both genes were down-regulated by RTV and it is therefore unlikely that they are involved in suppressing PPAR- γ . NRIP1 and RBL1 are involved in the conversion of WAT to a brown-fat-like phenotype and up-regulate BAT gene uncoupling protein-1 (UCP1) (Scime et al., 2005, Fritah et al., 2010). As such, these genes may be worth

investigating in future studies to determine the effects of ARVs on the conversion of WAT to BAT.

Mothers against decapentaplegic homolog 1 (SMAD1) was one of three genes in the adipocyte differentiation pathway up-regulated by RTV, a finding which has not been previously reported. SMAD1 is activated by bone morphogenic proteins and is responsible for activating PPAR- γ transcription (Jin et al., 2006). SMAD1 expression was up-regulated in our experiment, while PPAR- γ transcription was down-regulated, indicating that SMAD1 is unlikely to be involved in RTV-mediated PPAR- γ down-regulation. Similarly, PPAR- γ coactivator 1 alpha (PPARGC1) was up-regulated by RTV in our experiment, yet expression of PPAR- γ was down-regulated. PPARGC1 expression has been shown to be lower in SCAT biopsy samples from HALS patients compared with patients without HALS (Kannisto et al., 2003).

Additional genes altered in the 'Insulin, IGF-1 and TNF- α in brown adipocyte differentiation' pathway included adiponectin, insulin-like growth factor (IGF)-1, insulin receptor substrate (IRS)-2, beta 3 adrenergic receptor (ADRB3), GLUT4 and FASN. GLUT4 and FASN are well known to be down-regulated in response to RTV (Grigem et al., 2005) and explain in part the mechanisms underlying RTV-induced disturbances in adipocyte lipid and glucose metabolism. The *in vitro* effect of ARVs on IGF-1 has not yet been investigated, but serum IGF-1 was found to be higher in patients treated with PI compared with those treated with NNRTI and healthy subjects (Parfieniuk-Kowerda et al., 2013). Similarly, the *in vitro* effect of ARVs on the expression of ADRB3 has not previously been shown. Acute activation of ADRB3 is suggested to result in HSL-mediated release of FFA from adipocytes resulting in an inflammatory process which leads to down-regulation of PPAR- γ and CEBP- α (Mottillo et al., 2010). Moreover, Vonkeman (2000) showed that patients with a polymorphism in the ADRB3 gene were more likely to develop LD when taking ARVs compared with those who do not have the polymorphism. We have shown down-regulation of ADRB3 in response to RTV, which might be expected to result in increased PPAR- γ expression. However, as PPAR- γ was down-regulated by RTV in our experiment, it excludes the possibility that RTV may mediate its effect on PPAR- γ via ADRB3.

3.6.9 Limitations and conclusion

Limitations of our study include the fact that no combinations of ARV were tried in the experiments, as the focus was to elucidate the effects of each drug individually. As each drug was investigated separately, our experimental set-up does not represent the fixed drug combinations currently used *in vivo*. In the current study, we investigated the effects of RTV at near C_{\max} concentrations only. *In vivo*, levels of circulating ARVs are not sustained i.e. levels peak and trough. Furthermore, protein binding, blood flow and drug transporters may also play a key role in drug distribution, which will affect circulating ARV concentrations. RTV, which is routinely used as a boosting agent to increase the plasma concentrations of other PI (Hsu et al., 1998), appears to have a profound effect on adipocyte function and differentiation. Microarray analysis confirmed some of the RT-PCR data and identified additional genes modulated by RTV. We hypothesise that modulation of ERK1/2 signalling and LXR- α expression could represent key events leading to down-regulation of PPAR- γ and adipogenic genes, and inhibition of adipocyte differentiation and TG storage as observed in RTV treated cells. PPAR- γ is a nutrient sensor and master adipogenic transcription factor, and may therefore represent a suitable target for nutrient interventions to combat ARV-induced adipocyte dysfunction.

Chapter 4

The effects of conjugated linoleic acid on 3T3-L1 adipocytes pre-treated with antiretroviral drugs

4.1 Introduction

CLA isomers are positional and stereo-isomers of linoleic acid formed by bacterial biohydrogenation of linoleic acid in the rumen (Bauman et al., 1999). The c9,t11 and t10,c12 isomers appear to be the most biologically active (Kennedy et al., 2010). c9,t11 constitutes 90% of total dietary CLA and is found largely in beef and dairy products (Reynolds and Roche, 2010), while commercial CLA supplements typically contain large quantities of the t10,c12 isomer (Wang and Jones, 2004). CLA isomers may have beneficial effects with respect to obesity (Onakpoya et al., 2012), inflammation (Sofi et al., 2010), cancer (Mohammadzadeh et al., 2013), bone health (Deguire et al., 2012) and regression of atherosclerosis (Mitchell et al., 2012).

PPAR- γ is a master adipogenic transcription factor (Desvergne and Wahli, 1999) and its down-regulation has been suggested to be central to the development of HALS (Caron et al., 2009). Down-regulation of PPAR- γ has been demonstrated in numerous *in vitro* studies in response to ARVs (Caron et al., 2001, Bastard et al., 2002a, Caron et al., 2004, Grigem et al., 2005, Pacenti et al., 2006, Díaz-Delfín et al., 2011), as well as in mice (Goetzman et al., 2003). Furthermore, adipose tissue biopsies from HALS patients have shown reduced expression of PPAR- γ compared with non-lipodystrophic patients (Gallego-Escuredo et al., 2013). A study in healthy volunteers receiving NRTI for 2 weeks showed decreased adipose tissue expression of PPAR- γ (Mallon et al., 2005), while a 6-month interruption of HAART has been shown to improve adipose tissue expression of PPAR- γ (Kim et al., 2007c) indicating that changes in PPAR- γ , although quick to occur, may be reversible.

Binding of lipophilic ligands to PPAR- γ leads to the association of the transcription factor with co-activators and heterodimerisation with RXR. The heterodimer then targets the co-activator complex to PPARE on the target gene promoter, which initiates target gene transcription (Desvergne and Wahli, 1999). TZDs are synthetic PPAR- γ ligands, which are licensed for the treatment of T2DM and have been investigated for their role in mitigating aspects of HALS (Raboud et al., 2010). Pioglitazone was found to be effective at increasing limb fat in HIV-infected individuals compared with placebo (Raboud et al., 2010). Another study in patients with LA found that after only 2 weeks of rosiglitazone treatment, PPAR- γ expression in SCAT was significantly increased (Mallon et al., 2008). Current European AIDS Clinical Society guidelines for the management of diabetes in HIV question the use of TZDs and recommend metformin as

the first choice (European AIDS Clinical Society, 2014). These findings suggest that PPAR- γ ligands may be potentially beneficial in the management of aspects of HALS, but due to questions regarding the safety of TZDs, natural ligands should be explored.

CLA isomers are natural PPAR- γ ligands (Belury et al., 2002, Brown et al., 2003) and have been shown to differentially affect adipocyte lipid accumulation (Granlund et al., 2003), adiponectin secretion (Ahn et al., 2006) and expression and secretion of perilipin (Chung et al., 2005). As far as could be ascertained, no studies have investigated the effects of CLA isomers on PPAR- γ and target genes in adipocytes exposed to ARVs.

4.2 Hypothesis

CLA isomers, as PPAR- γ ligands, will mitigate adipocyte dysfunction, PPAR- γ down-regulation and modulate the inflammatory profile in 3T3-L1 adipocytes exposed to ARVs.

4.3 Aims

The specific aims of this study were to investigate the effects of two CLA isomers (c9,11 and t10,c12) on:

- adipocyte function (TG accumulation)
- expression of markers of adipocyte differentiation (PPAR- γ , adiponectin, perilipin, Cidea)
- expression of markers of inflammation (IL-6, leptin, resistin)
- global gene expression

in 3T3-L1 adipocytes exposed to ARVs.

4.4 Methods

4.4.1 Experimental model

The 3T3-L1 murine adipocyte cell line was maintained as outlined in **Section 2.1.3**. Cells were enumerated by Trypan Blue staining and seeded at a density of 5×10^5 cells in 75 ml tissue culture flasks. Once confluent, cells were sub-cultured, enumerated and re-seeded at a density of 2.4×10^5 cells in 6-well plates and 0.6×10^5 cells in 24-well plates. Cells were grown to 80-90% confluence in induction medium (day 1-5) and incubated for a further 48 hours to initiate growth arrest. On day 7, the medium was removed and replaced with adipogenic medium consisting of induction medium plus IBMX, dexamethasone and insulin. After two days, (day 9) this medium was removed and replaced with new medium containing insulin only, ARVs which showed significant effects in Chapter 3, as well as CLA isomers. Differentiated adipocytes were treated with near C_{\max} concentrations of RTV (20 μM), IDV (10 μM) or TDF (1 μM), in addition to 100 μM c9,t11 or t10,c12 for 5 days in 6- or 24-well plates, as previously described. CLA isomers, c9,t11 and t10,c12, were dissolved in vehicle control DMSO. All treated cells received one or both vehicles consisting of 0.1% ethanol and/or 0.1% DMSO as appropriate. Thereafter, the medium was changed every 2 days until cells were fully differentiated at day 14. Cells were exposed to the respective treatments for a total of five days. All experiments were conducted on mature adipocytes at day 14.

4.4.2 Oil Red O staining

Once adipocytes reached maturity (day 14), induction medium was removed and cells were washed once with PBS. Cells were formalin fixed for 1 hour. Formalin was removed, allowed to dry and the cells were washed with 60% isopropanol. Isopropanol was removed and cells were incubated in ORO working solution for 10 minutes. The ORO was removed and the cells were washed four times with distilled water. The ORO was then eluted by incubating in 100% isopropanol for 10 minutes. A volume of this solution was then transferred to a 96-well plate and the optical density of the solution was measured as described in **Section 2.1.9**.

4.4.3 Real time RT-PCR

At day 14, total RNA was extracted from adipocytes using the RNeasy Mini Kit as described in **Section 2.1.10**. Briefly, cells were lysed and homogenised in the presence of a highly denaturing guanidine-thiocyanate-containing buffer to inactivate RNases and ensure purification of intact RNA. Ethanol was added to provide appropriate binding conditions, and the sample was then applied to an RNeasy Mini spin column, where total RNA binds to the membrane and contaminants were efficiently washed away. High-quality RNA was then eluted in RNase-free water. RNA was quantified using the NanoDrop[®] spectrophotometer. cDNA fragments were generated by reverse transcription using the High Capacity RNA-to-cDNA kit as described in **Section 2.1.12**. TaqMan[®] RT-PCR was performed for PPAR- γ , adiponectin, perilipin, Cidea, leptin, resistin, IL-6 and Hprt (endogenous housekeeping control) using pre-developed assay kits and the ABI Prism 7000 sequence detection system (see **Section 2.1.13**).

4.4.4 Sandwich ELISA

On day 14, culture medium was removed and centrifuged at 5,000 rpm for 10 minutes. Supernatants were removed from the resulting adipocyte pellets and stored at -80 °C. Secretion of adiponectin into supernatant was quantified by DuoSet ELISA as outlined in **Section 2.1.15.1**.

4.4.5 PPAR- γ consensus site binding

Nuclear activation of PPAR- γ (binding to PPRE) was investigated using a DNA binding ELISA TransAM[™] PPAR- γ Transcription Factor Assay Kit (Active Motif) (**Section 2.1.18**). Briefly, complete binding buffer was added to each well of a 96-well microplate to which the PPRE consensus sequence (5'-AACTAGGNCAAAGGTCA-3') was immobilised. 10 μ g nuclear extract from ARV and ethanol treated cells was diluted in lysis buffer and added to each well. The plate was incubated for one hour at room temperature with gentle agitation. Primary antibody was directed against PPAR- γ and a secondary anti-mouse IgG antibody conjugated with HRP were added to each well. Absorbance was read in a spectrophotometer at 450 nm with a reference wavelength of 655 nm.

4.4.6 Global gene expression assay

Microarray analysis was carried out on RTV-treated adipocytes, as RTV emerged as the most potent ARV from Chapter 3. RNA extracted from adipocytes treated with control (RTV + DMSO), RTV + c9,t11 or RTV + t10,c12 were analysed to provide support for the RT-PCR data and to identify any additional genes and pathways which might be affected by the addition of CLA to RTV treated cells. On day 14, total RNA was extracted from adipocytes using the RNeasy Mini Kit as described in **Section 2.1.10**. RNA samples were prepared for microarray analysis using MessageAmp™ Premier RNA Amplification Kit as described in **Section 2.1.14**. RNA samples were pooled and 500 ng of each pooled sample in 5 µl was used in the reactions. The concentration of the resulting RNA was determined using the NanoDrop® spectrophotometer. Biotinylated aRNA was fragmented prior to hybridisation using the 5X Array Fragmentation Buffer supplied with the MessageAmp™ Kit. The sample was then evaluated using the Agilent® 2100 Bioanalyser instrument and RNA 6000 Nano Kit loaded with 300 ng of aRNA per well. 11 µg of aRNA were hybridised onto the Affymetrix Mouse Genome 430 2.0 Array Kit (Affymetrix, UK) and were subsequently washed, stained and scanned on the Fluidics Station 400 and the GeneArray Scanner (Affymetrix, UK) according to the manufacturer's protocol. Data were analysed using the RMA algorithm and the average change in expression (ratio of treatment/ control) determined for each gene. Metacore™ pathway analysis software (GeneGo, Thomson Reuters) was used for functional analysis of differentially expressed genes and pathway analysis.

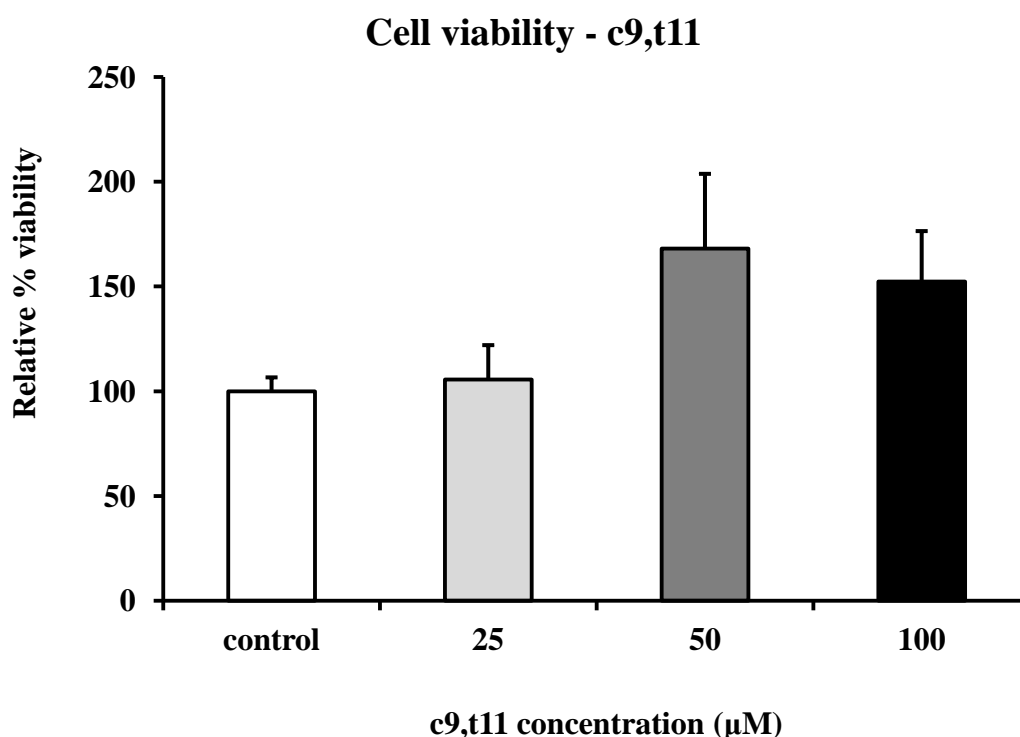
4.4.7 Statistical analysis

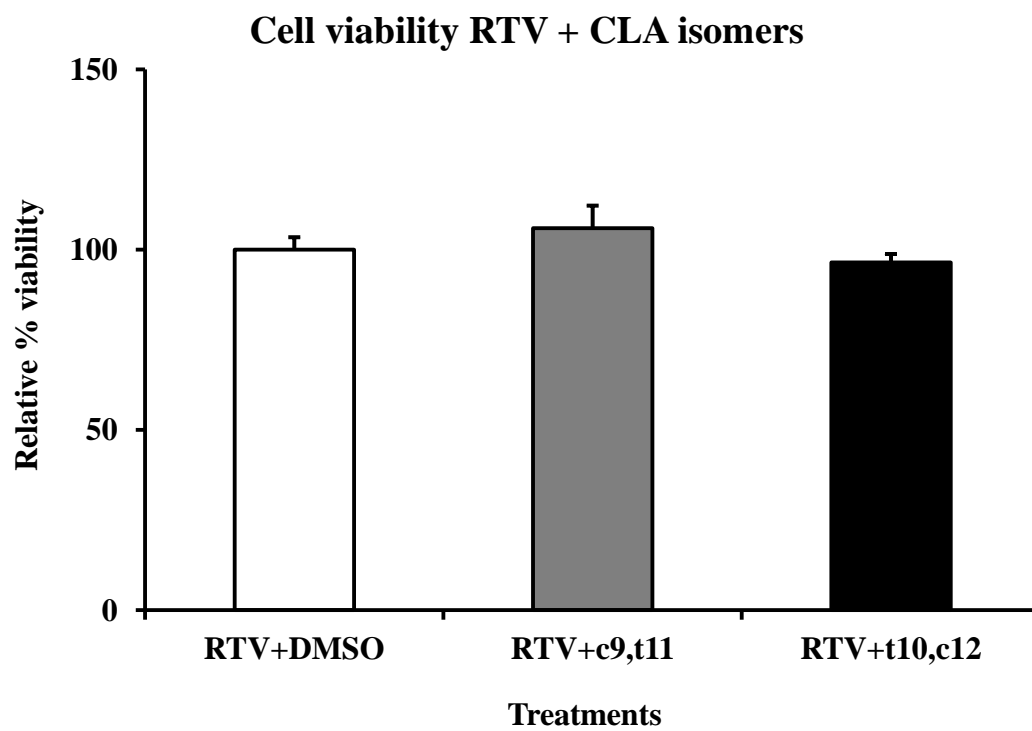
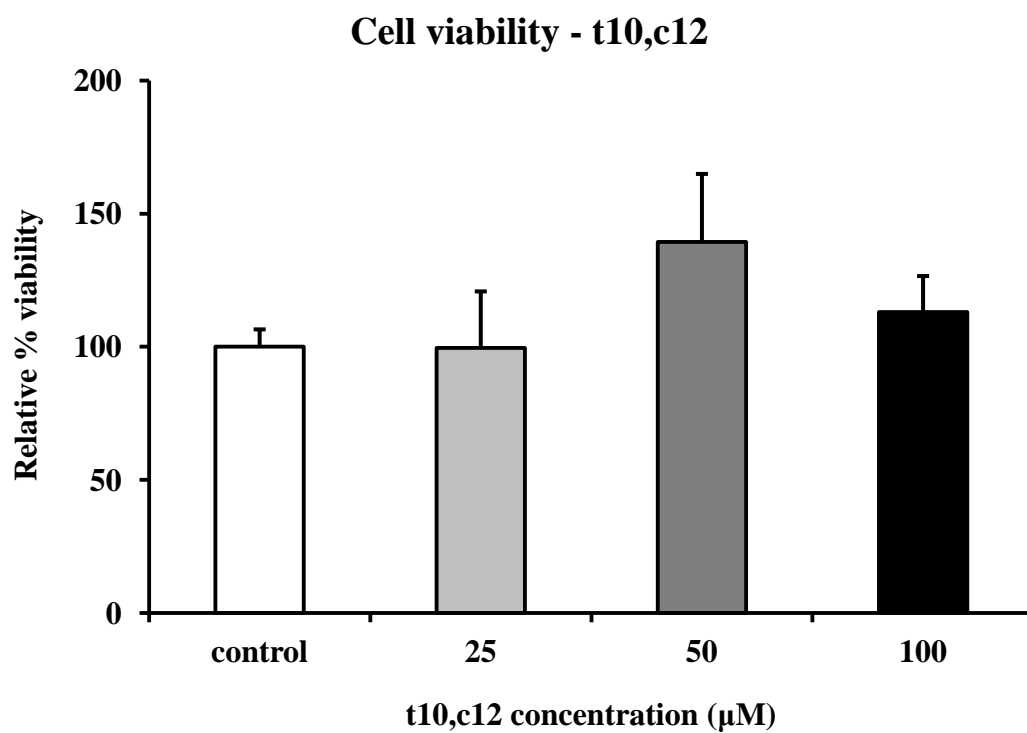
Statistical analyses were performed using SPSS software (version 20; IBM Corp). Data were analysed using one way analysis of variance (ANOVA), with post-hoc analysis using Dunnett's test after demonstration of significant differences by ANOVA. Differences were considered significant if $P \leq 0.05$. Data are expressed as the mean \pm SEM of four independent observations unless otherwise stated. For microarray analysis, a 1.5-fold (signal log ratio ≥ 0.5 or ≤ -0.5) and 2-fold (signal log ratio ≥ 1.0 or ≤ -1.0) change relative to control was selected as the threshold to define differentially expressed genes in c9,t11 and t10,c12 treated cells, respectively.

4.5 Results

4.5.1 Cell viability

Adipocyte viability was assessed at day 14 using an MTS assay. Increasing concentrations of CLA isomers (25 μ M, 50 μ M and 100 μ M) were tried and compared with vehicle control (DMSO). There was no significant difference in cell viability between any concentration of c9,t11 (**Figure 4.1 A**) or t10,c12 (**Figure 4.1 B**). As a result, it was decided that a concentration of 100 μ M would be used for all experiments, as this concentration of c9,t11 and t10,c12 was used previously in 3T3-L1 adipocytes (Ahn et al., 2006). We then investigated cell viability in adipocytes treated with ARVs found to have significant effects in Chapter 3, RTV, IDV and TDF, and CLA isomers, and found no significant difference in cell viability between cells treated with control and near C_{\max} ARVs + 100 μ M CLA isomers (**Figure 4.1 C, D and E**).





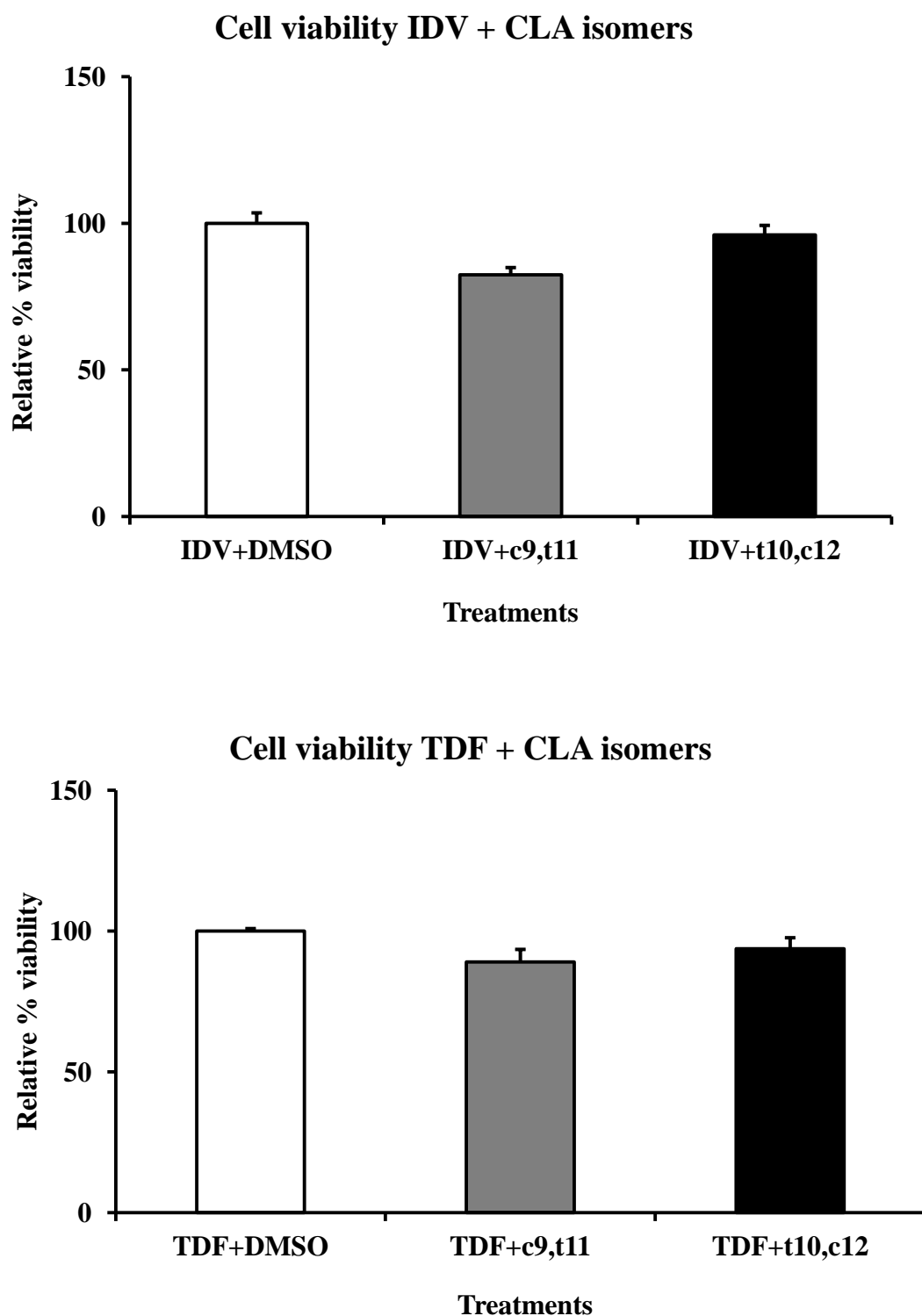


Figure 4.1 Effect of ARVs and CLA on cell viability. Adipocytes were treated with (A) vehicle control (DMSO) or c9,t11 and (B) vehicle control (DMSO) or t10,c12. 100 μ M c9,t11 or t10,c12 were then added to cells treated with near C_{max} concentrations of (C) RTV, (D) IDV or (E) TDF and compared with ARV + vehicle control. An MTS assay was carried out at day 14 to determine cell viability, which was measured spectrophotometrically at 490 nm. Results represent mean \pm SEM of 4 parallel cell culture experiments. P for ANOVA = 0.880, 0.966, 0.331, 0.389 and 0.133, respectively.

4.5.2 Triglyceride accumulation

RTV was the only ARV to significantly alter TG accumulation in previous experiments (Section 3.5.2) and as a result, the effect of the two CLA isomers on TG accumulation was investigated in adipocytes exposed to RTV. Under RTV exposure, both c9,t11 and t10,c12 significantly increased TG accumulation compared to RTV-treated control cells ($P < 0.001$ for both; Figure 4.2). The effect of RTV and CLA isomers on TG accumulation is illustrated in Figure 4.3.

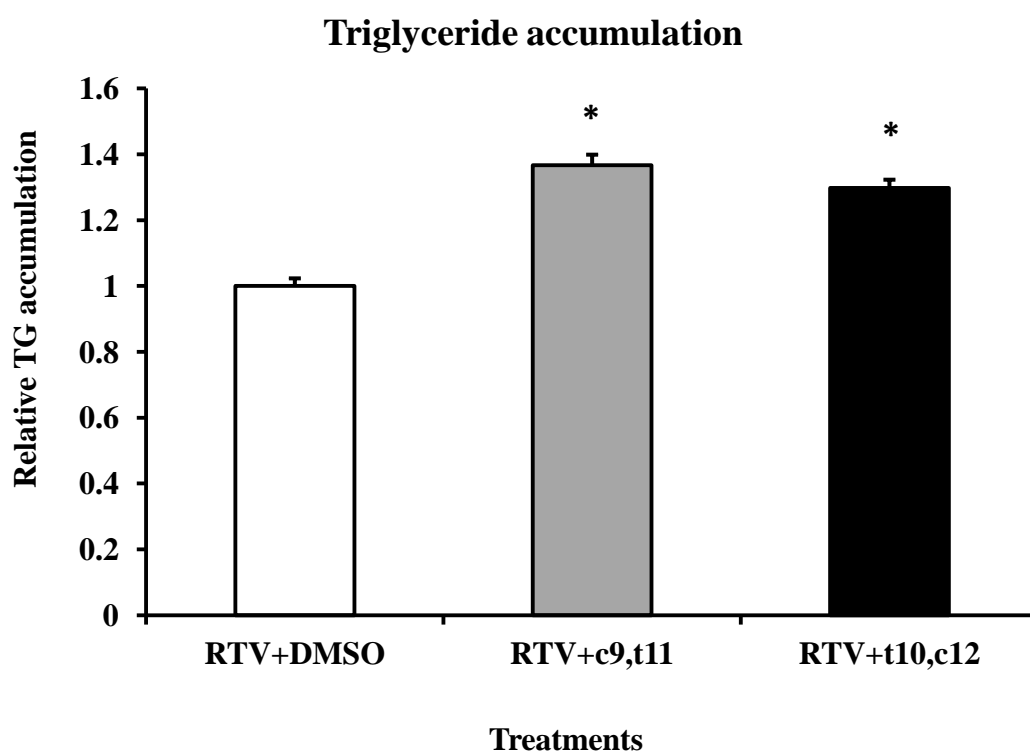
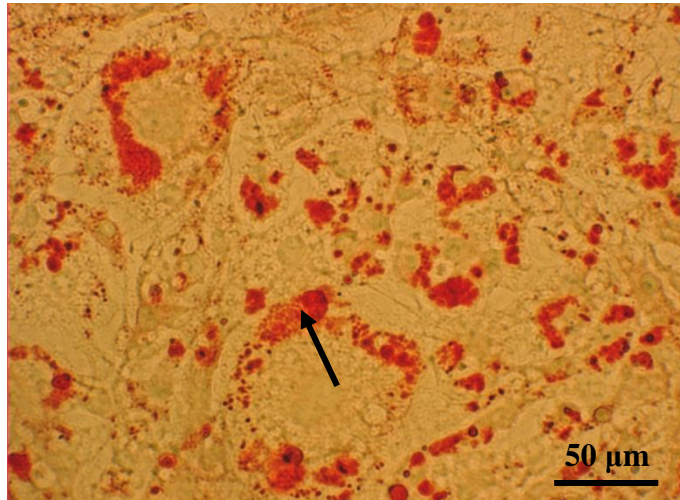
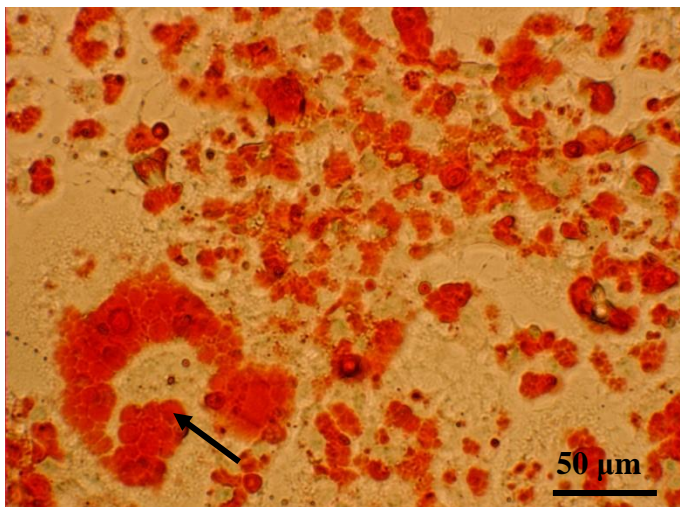


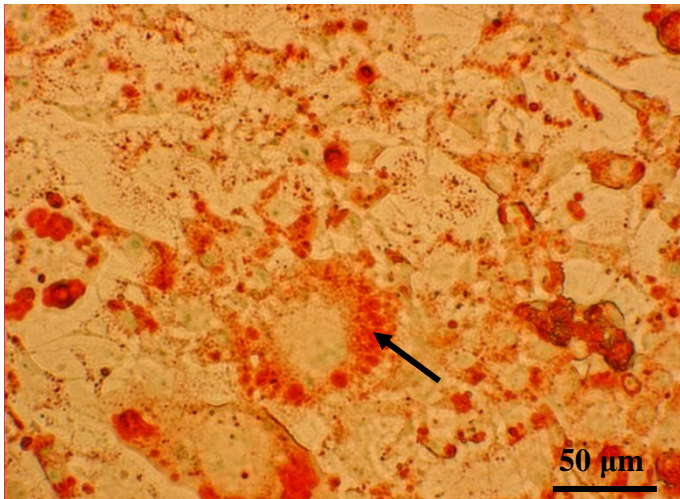
Figure 4.2 Effect of RTV and CLA on TG accumulation. Adipocytes were treated with control (RTV + DMSO) or near C_{\max} concentrations of RTV and 100 μM of c9,t11 or t10,c12. Adipocytes stained at day 14 with ORO to quantify lipid content, which was measured spectrophotometrically at 540 nm. Results represent mean \pm SEM of 8 parallel cell culture experiments. *Significantly different ($P < 0.001$) from control cells.



RTV + DMSO



RTV + c9,t11



RTV + t10,c12

Figure 4.3 Oil Red O stained adipocytes treated with RTV and CLA. Adipocytes were treated with control (RTV + DMSO) or near C_{\max} concentrations of RTV and 100 μM of c9,t11 or t10,c12. Adipocytes were stained at day 14 with ORO to visualise lipid content. Black arrows indicate cells with lipid droplets and lipid vesicles (magnification is at 40x).

4.5.3 Lipid droplet protein transcription

RTV was the only ARV which significantly altered perilipin expression in previous experiments (**Section 3.5.3**). The effect of CLA isomers on the expression of genes for lipid droplet-associated protein perilipin was then investigated in 3T3-L1 adipocytes exposed to RTV. Under RTV exposure, t10,c12 significantly decreased perilipin gene expression compared with control ($P = 0.004$; **Figure 4.4**). There was no significant effect of c9,t11.

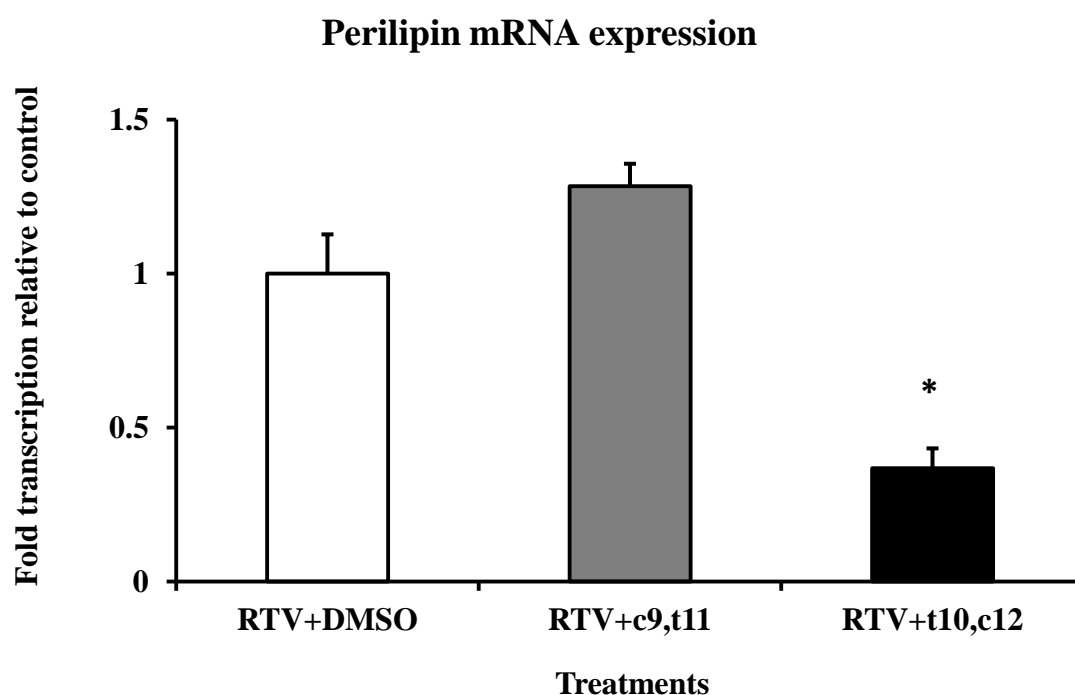
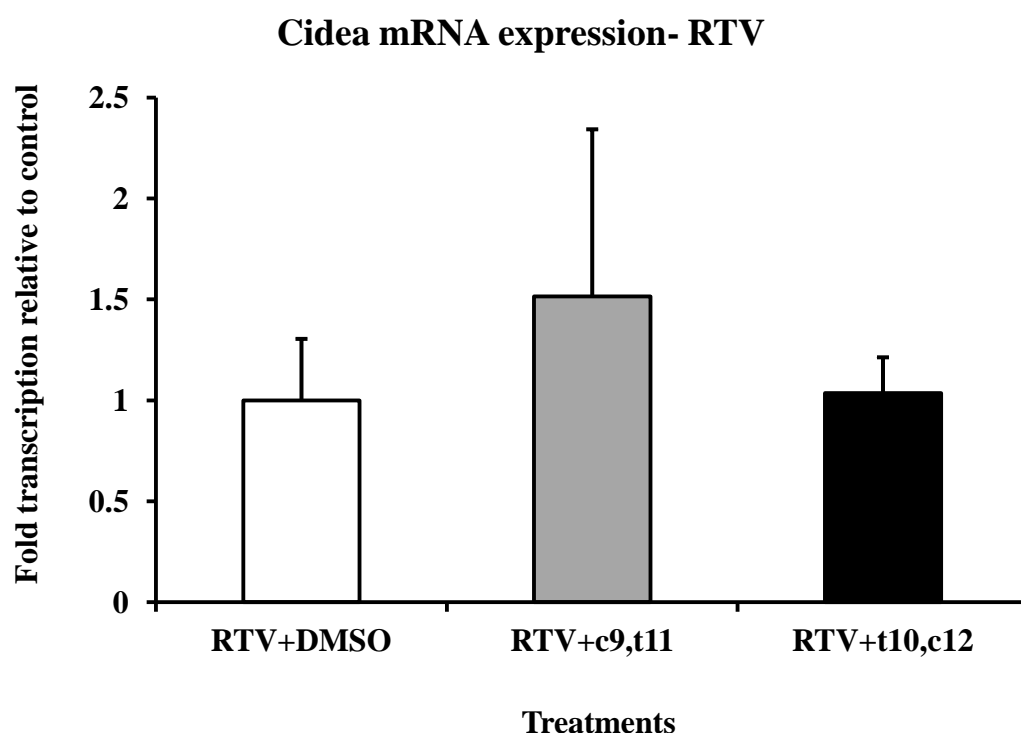


Figure 4.4 Effect of RTV and CLA on perilipin transcription. Adipocytes were treated with control (RTV + DMSO) or near C_{\max} concentrations of RTV and 100 μM of each CLA isomer. Expression of perilipin mRNA was measured on day 14 relative to Hprt housekeeping gene by RT-PCR. Results represent mean \pm SEM of 4 parallel cell culture experiments. *Significantly different ($P = 0.004$) from control cells.

RTV, IDV and TDF were the only ARVs to significantly affect Cidea transcription in previous experiments (**Section 3.5.3**) and as a result the effect of CLA isomers on the expression of Cidea was tested in adipocytes treated with these three ARVs. CLA isomers had no significant effect on Cidea expression in RTV-treated cells (**Figure 4.5 A**). In IDV- and TDF-treated cells, t10,c12 significantly decreased Cidea gene expression compared with control ($P = 0.003$ and $P = 0.012$, respectively; **Figure 4.5 B and C**). c9,t11 had no significant effect in IDV and TDF treated cells.

A



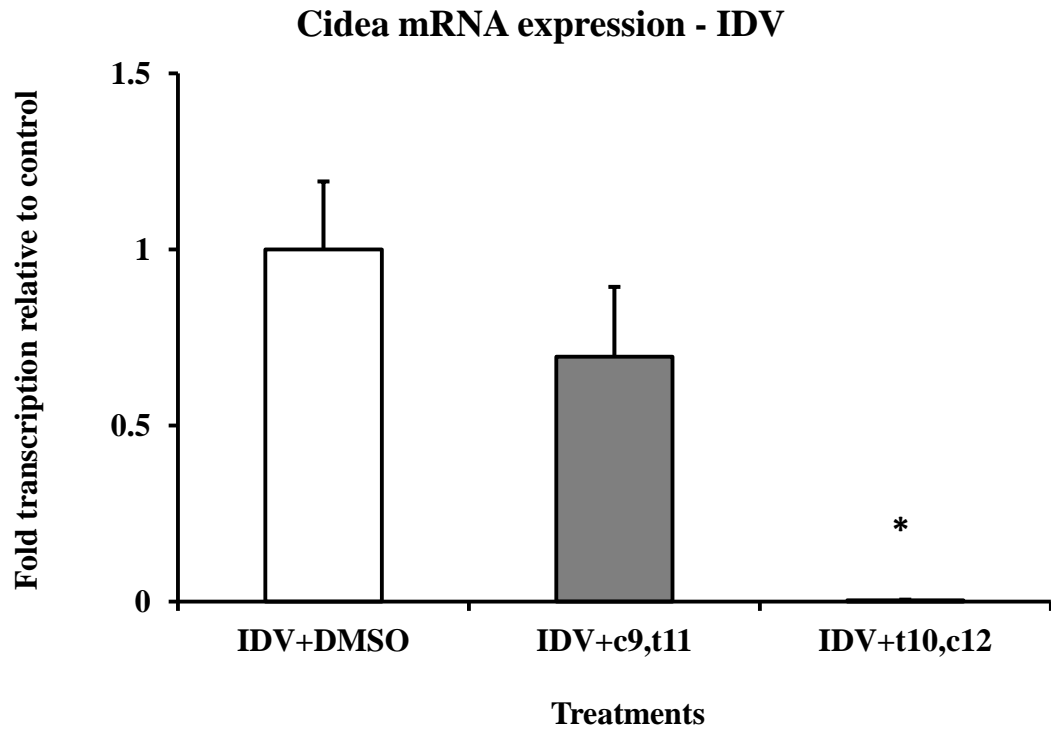
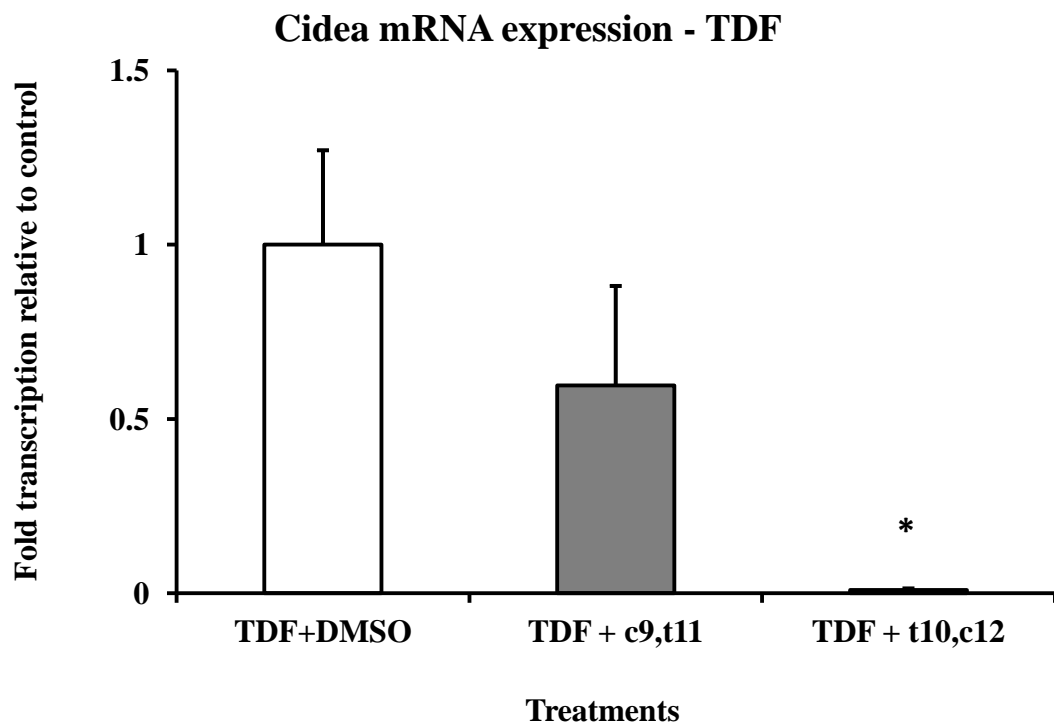
B**C**

Figure 4.5 Effect of ARVs and CLA on Cidea transcription. Adipocytes were treated with control or near C_{max} concentrations of (A) RTV, (B) IDV or (C) TDF and 100 μ M of c9,t11 or t10,c12. Expression of Cidea mRNA was measured on day 14 relative to Hprt housekeeping gene by RT-PCR. Results represent mean \pm SEM of 4 parallel cell culture experiments. *Significantly different ($P = 0.003$ and $P = 0.012$, respectively) from control cells.

4.5.4 PPAR- γ gene expression and nuclear activation

The effect of each CLA isomer on PPAR- γ gene expression was tested in RTV-treated cells, as RTV was the only ARV to significantly alter PPAR- γ gene expression in previous experiments (Section 3.5.4). Neither CLA isomer had a significant effect on PPAR- γ gene expression in RTV-treated cells (Figure 4.6).

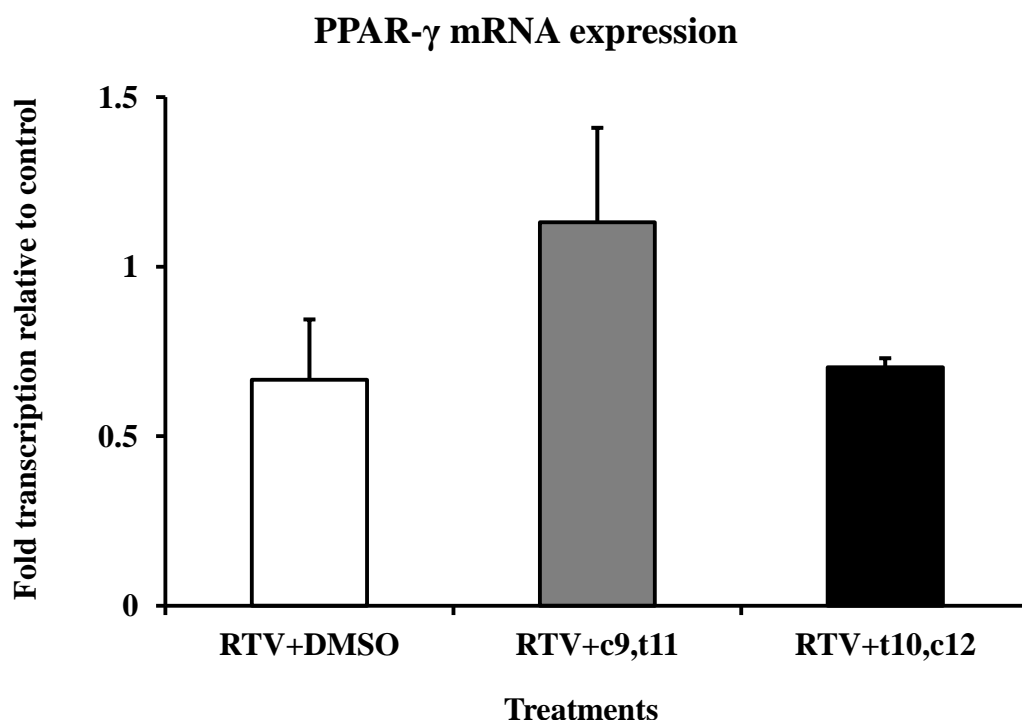
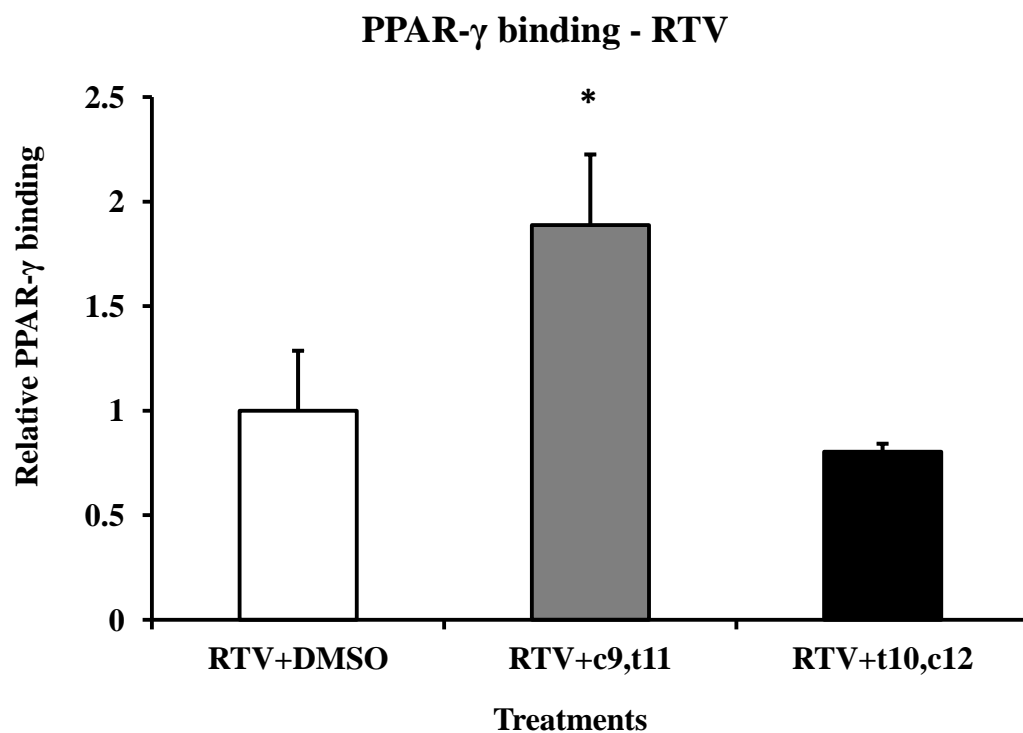


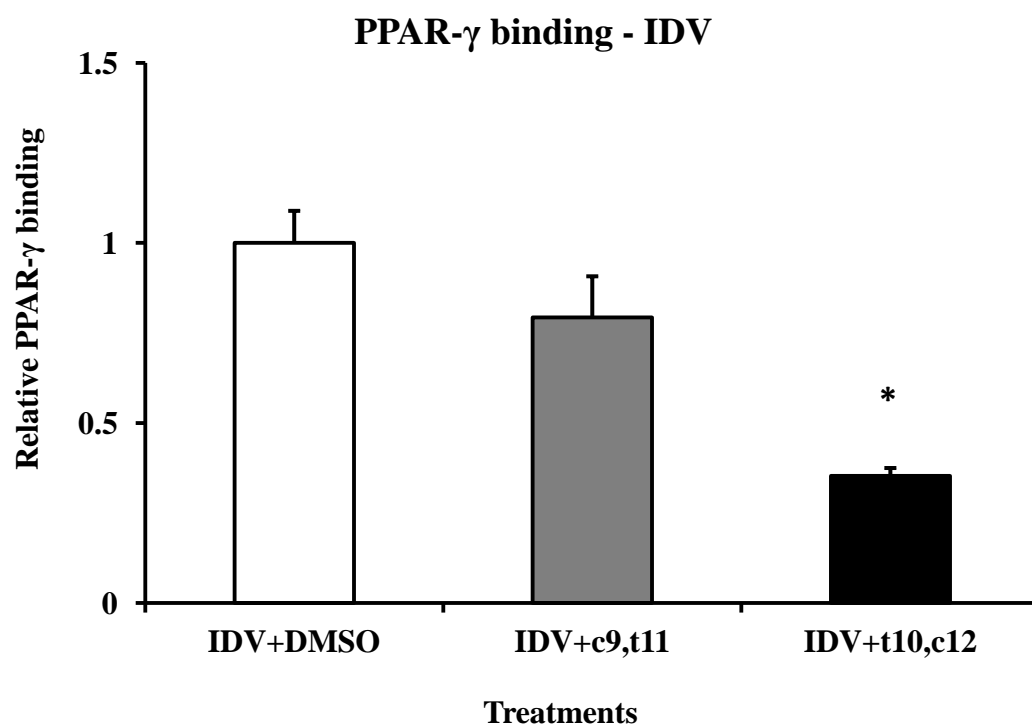
Figure 4.6 Effect of RTV and CLA on PPAR- γ transcription. Adipocytes were treated with near C_{\max} concentrations of RTV and DMSO or RTV and 100 μ M of c9,t11 or t10,c12. Expression of PPAR- γ mRNA was measured on day 14 relative to Hprt housekeeping gene by RT-PCR. Results represent mean \pm SEM of 4 parallel cell culture experiments.

The effect of CLA isomers on nuclear PPAR- γ binding to the immobilised consensus site (PPRE) was investigated in adipocytes treated with RTV, IDV and TDF, the ARVs shown to significantly affect PPAR- γ nuclear binding in previous experiments (Section 3.5.4). c9,t11 significantly increased nuclear PPAR- γ binding to the immobilised consensus site in both RTV- and TDF-treated cells ($P = 0.038$ and $P = 0.043$, respectively; Figure 4.7 A and C). t10,c12 significantly decreased nuclear PPAR- γ binding to the immobilised consensus site in IDV-treated cells ($P = 0.001$; Figure 4.7 B).

A



B



C

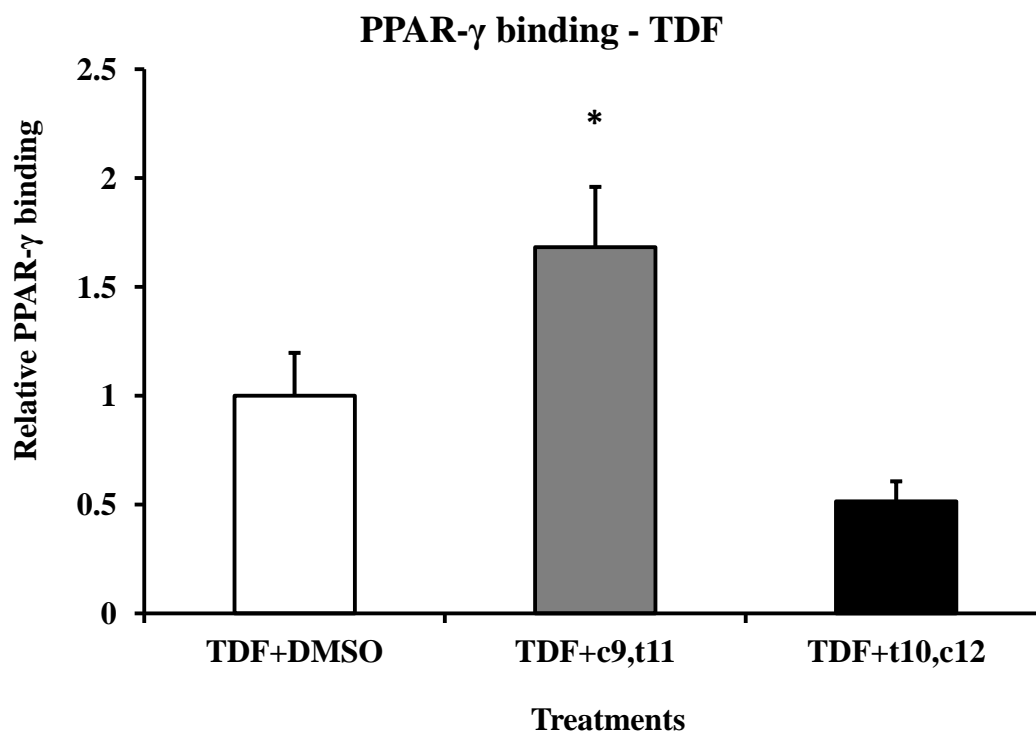
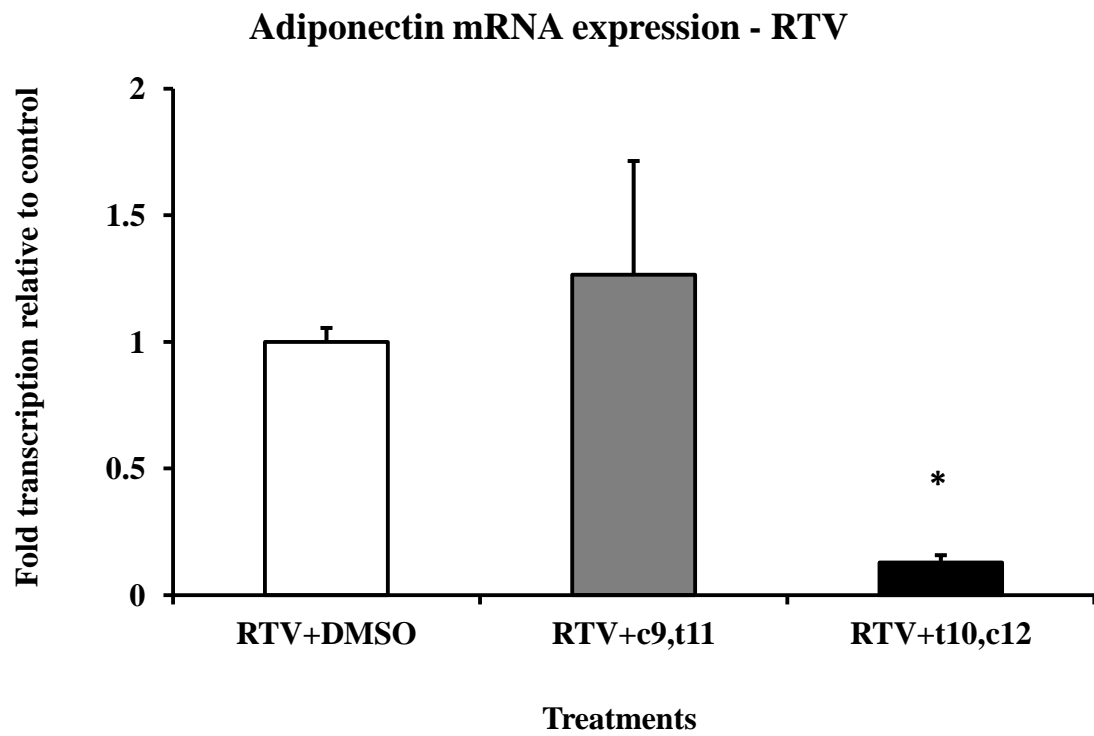


Figure 4.7 Effect of ARVs and CLA on nuclear PPAR- γ binding to PPRE. Nuclear proteins were extracted on day 14 from adipocytes treated with control or near C_{\max} concentrations of (A) RTV, (B) IDV, or (C) TDF and 100 μ M of c9,t11 or t10,c12 and nuclear PPAR- γ consensus site binding was measured. Results represent mean \pm SEM of 4 parallel cell culture experiments. *Significantly different ($P = 0.038$, 0.001 and 0.043, respectively) from control cells.

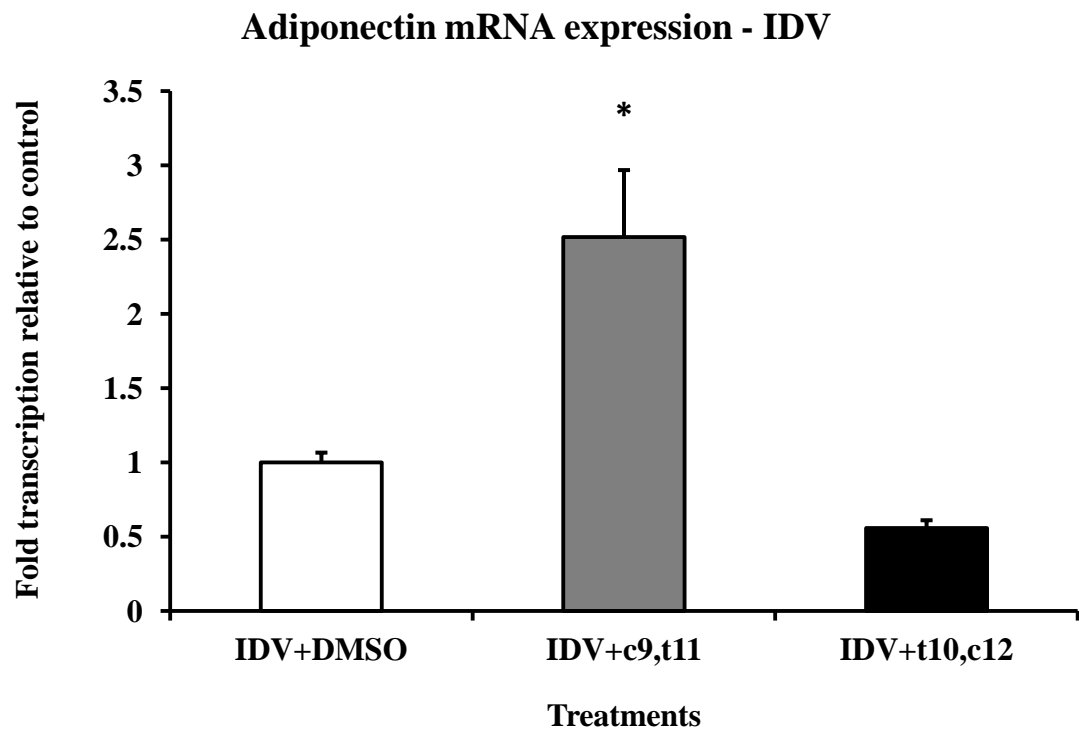
4.5.5 Adiponectin gene expression and secretion

Although d4T significantly altered adiponectin gene expression in previous experiments, it was thought that outliers in the data were obscuring the effect of the other drugs. For this reason, we examined adiponectin expression in adipocytes treated with CLA isomers and RTV, IDV or TDF only. In RTV-treated cells, c9,t11 had no significant effect on adiponectin gene expression, while t10,c12 caused a significant reduction in adiponectin expression ($P = 0.038$; **Figure 4.8 A**). c9,t11 significantly increased adiponectin gene expression in IDV-treated cells ($P = 0.005$; **Figure 4.8 B**). t10,c12 significantly decreased adiponectin gene expression in TDF-treated cells ($P < 0.001$; **Figure 4.8 C**).

A



B



C

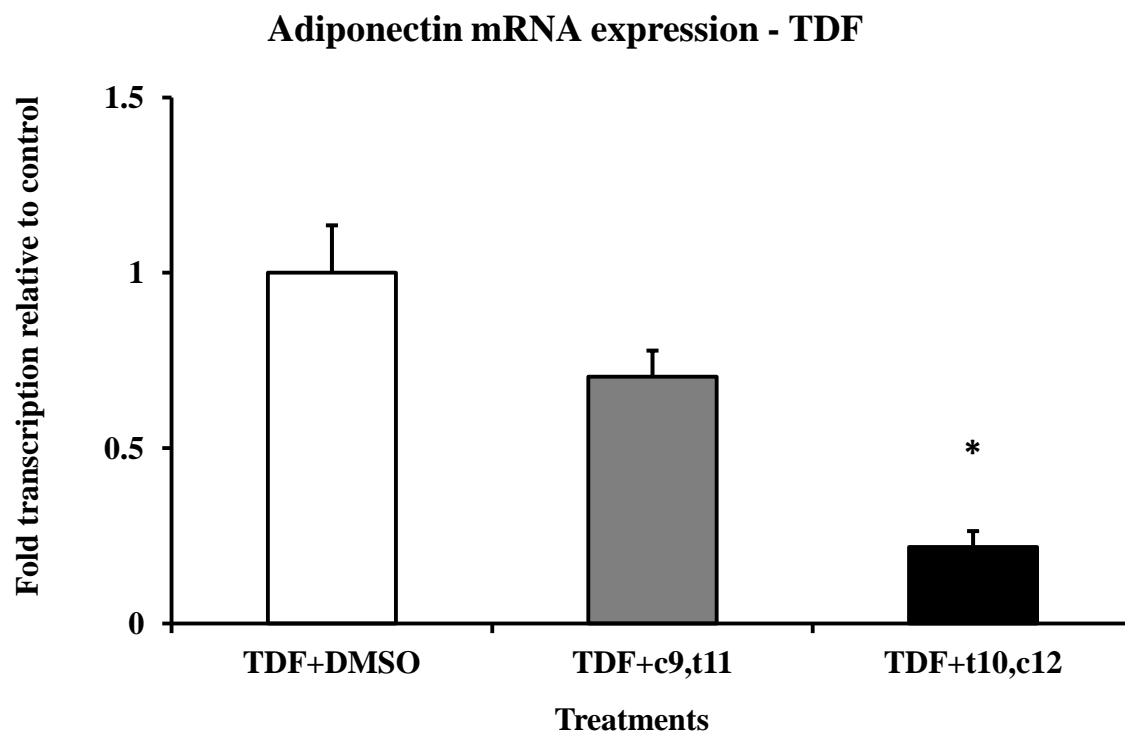
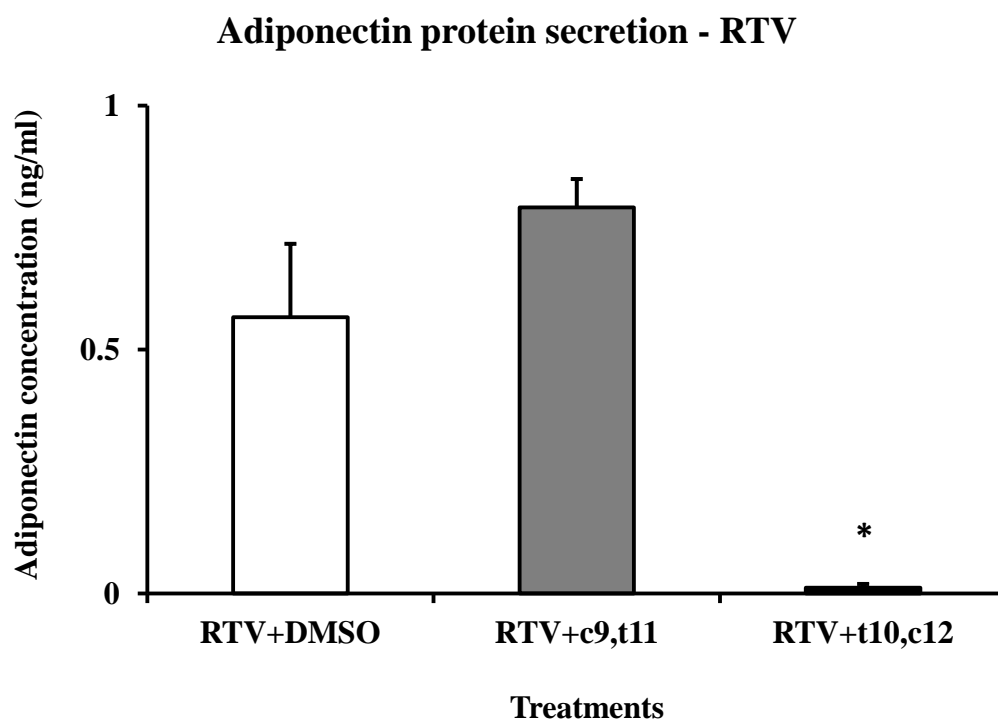


Figure 4.8 Effect of ARVs and CLA on adiponectin transcription. Adipocytes were treated with control or near C_{\max} concentrations of (A) RTV, (B) IDV or (C) TDF and 100 μ M CLA isomers, c9,t11 or t10,c12. Expression of adiponectin mRNA was measured on day 14 relative to Hprt housekeeping gene by RT-PCR. Results represent mean \pm SEM of 4 parallel cell culture experiments. *Significantly different ($P = 0.038$, 0.005 and < 0.001) from control cells.

The effect of the CLA isomers on adiponectin secretion was investigated in adipocytes treated with RTV, IDV and TDF, which had significant effects on adiponectin in previous experiments (**Section 3.5.5**). Although c9,t11 did not have a significant effect on adiponectin secretion in RTV-treated cells, the t10,c12 isomer significantly decreased its secretion ($P = 0.003$; **Figure 4.9 A**). Similarly, in IDV-treated cells t10,c12 decreased adiponectin secretion ($P < 0.001$; **Figure 4.9 B**). In TDF-treated cells, both isomers significantly decreased adiponectin protein secretion in ($P < 0.001$, both; **Figure 4.9 C**).

A



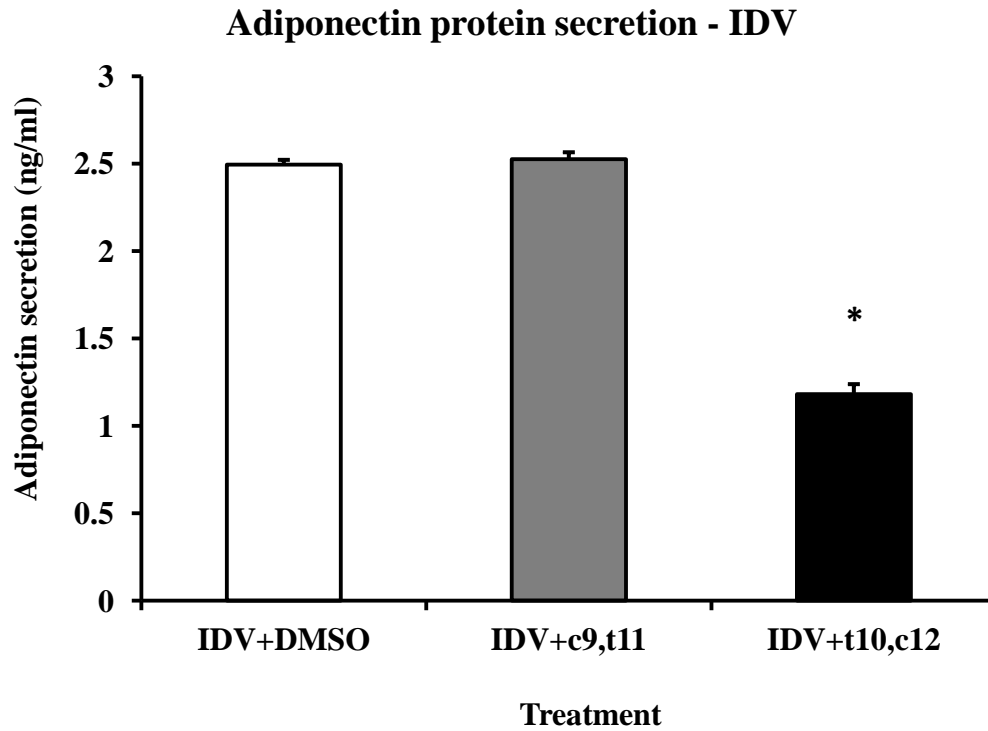
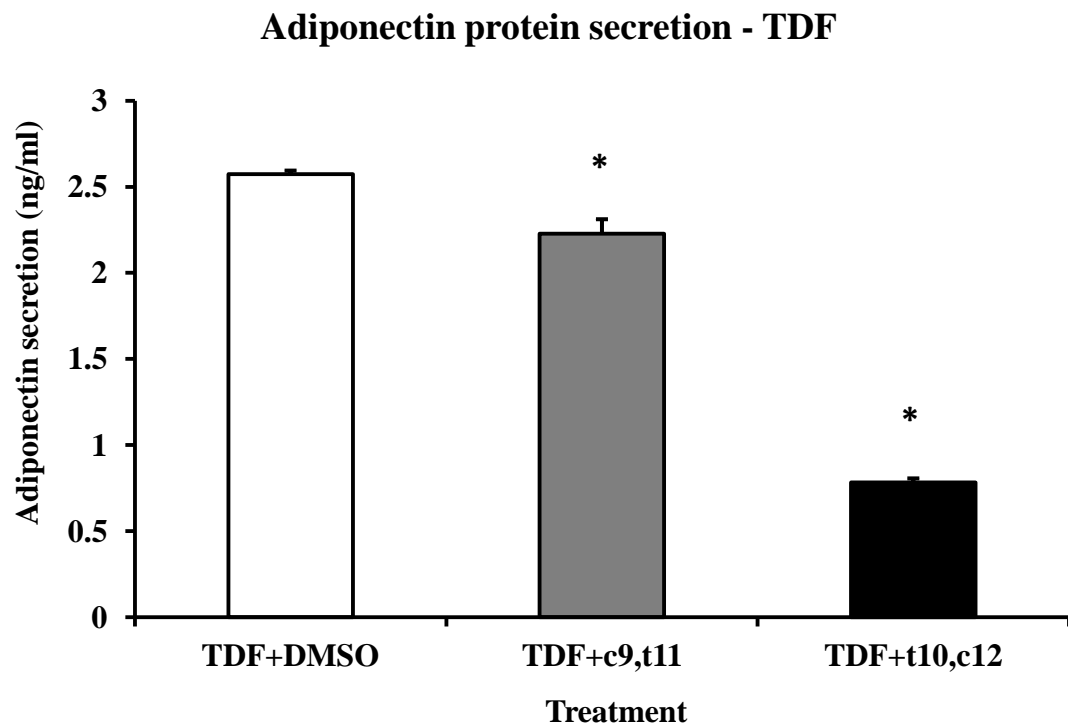
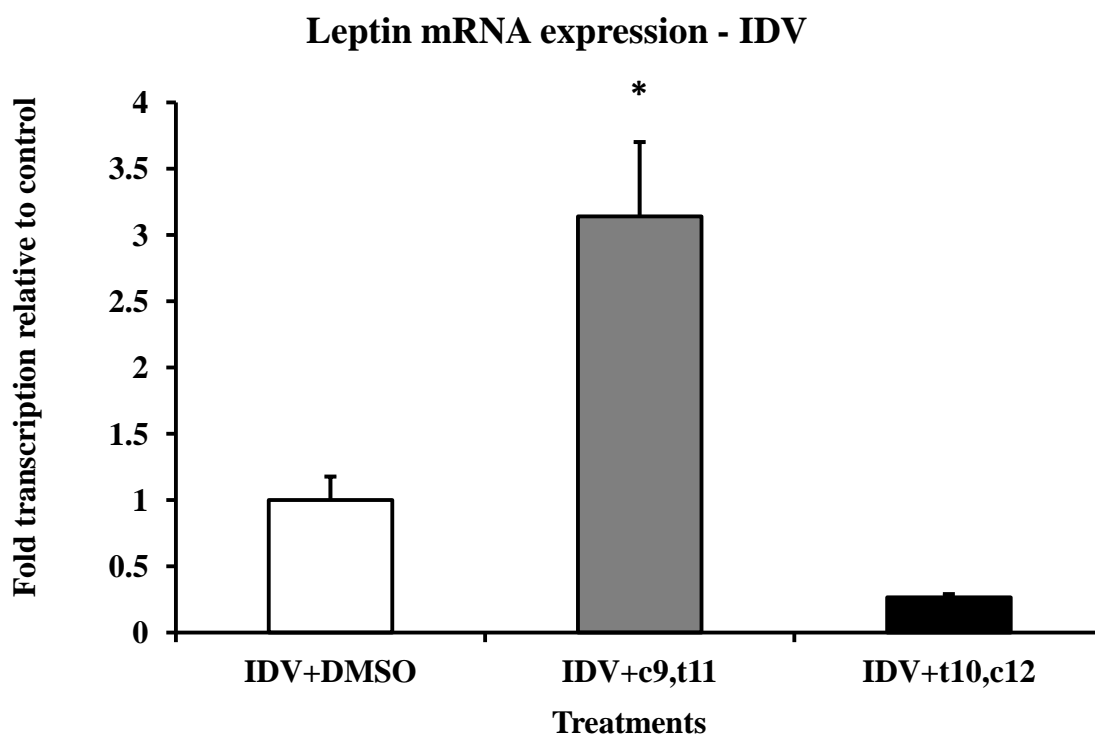
B**C**

Figure 4.9 Effect of ARVs and CLA on adiponectin protein secretion. Adipocytes were treated with control or near C_{max} concentrations of (A) RTV, (B) IDV or (C) TDF and 100 μ M CLA isomers, c9,t11 or t10,c12. Supernatants were collected from adipocytes on day 14 and used to quantify adiponectin secretion. Results represent mean \pm SEM of 4 parallel cell culture experiments. *Significantly different ($P = 0.003$, < 0.001 and < 0.001 , respectively) from control cells.

4.5.6 Leptin, resistin and IL-6 transcription

The effect of CLA isomers on adipokine expression was investigated in adipocytes treated with IDV and TDF, both of which had a significant effect on adipokine expression in previous experiments (**Section 3.5.6**). c9,t11 significantly increased leptin expression in IDV- and TDF-treated adipocytes ($P = 0.003$ and $P = 0.016$; **Figure 4.10**). t10,c12 had no significant effect.

A



B

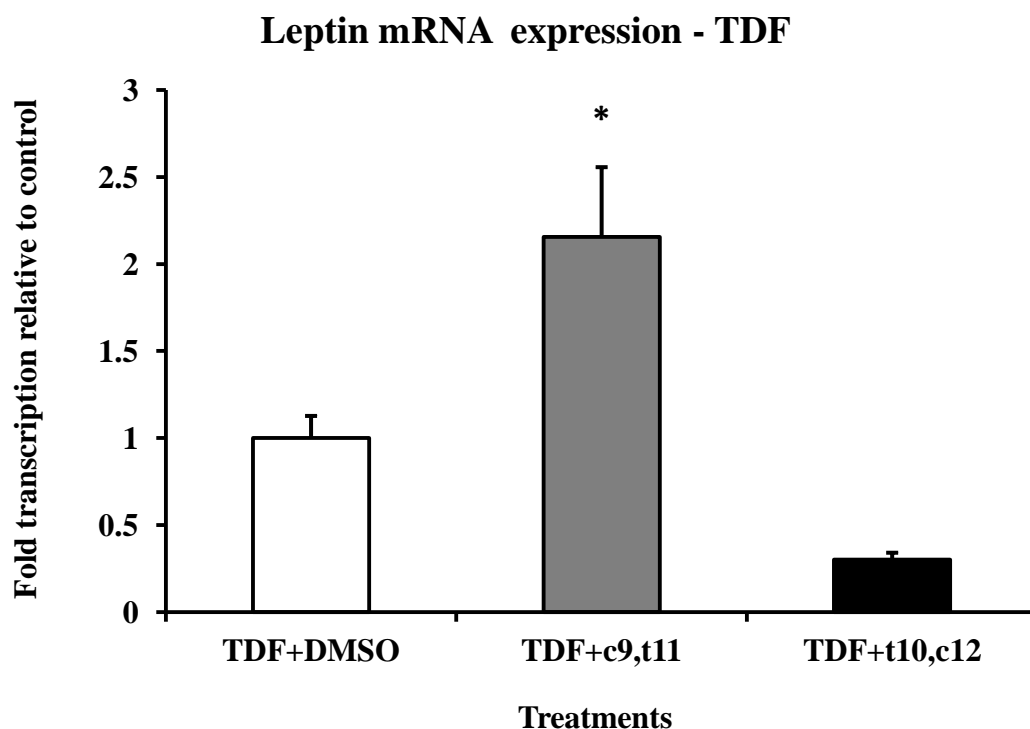


Figure 4.10 Effect of ARVs and CLA on leptin transcription. Adipocytes were treated with control, near C_{\max} concentrations of (A) IDV or (B) TDF and 100 μM CLA isomers, c9,t11 or t10,c12. Expression of leptin mRNA was measured on day 14 relative to Hprt housekeeping gene by RT-PCR. Results represent mean \pm SEM of 4 parallel cell culture experiments. *Significantly different ($P = 0.003$ and $P = 0.016$, respectively) from control cells.

The effect of CLA isomers on resistin gene expression was investigated in adipocytes treated with TDF, the ARV which had a significant effect on resistin expression in previous experiments (**Section 3.5.6**). The addition of c9,t11 had no significant effect on resistin expression in adipocytes treated with TDF, while t10,c12 significantly decreased resistin gene expression ($P = 0.033$; **Figure 4.11**).

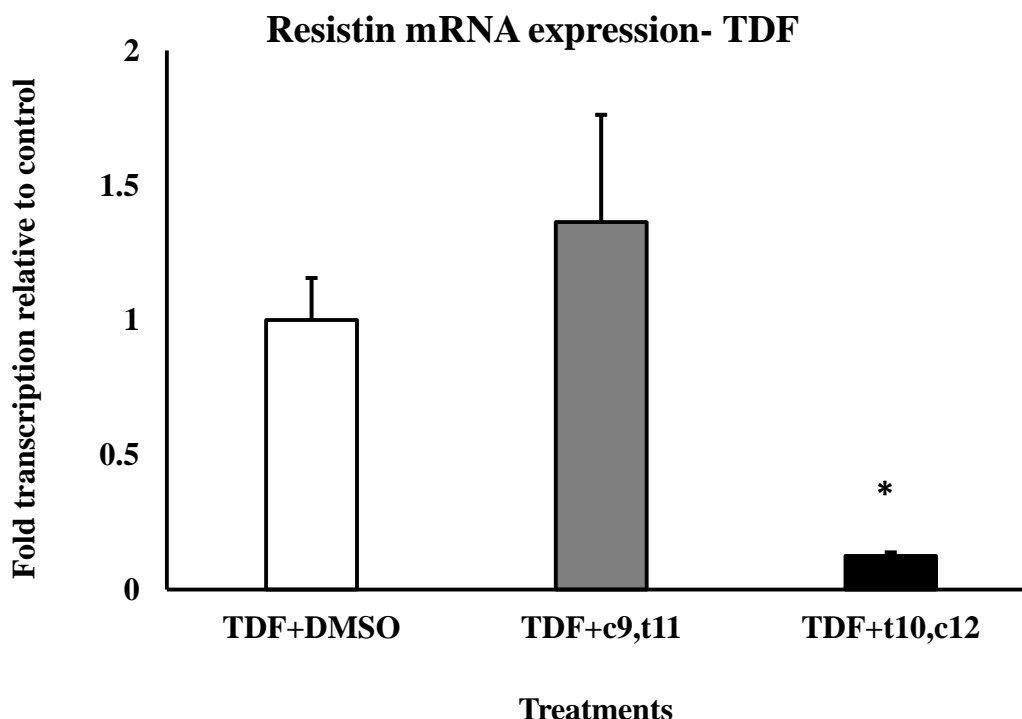
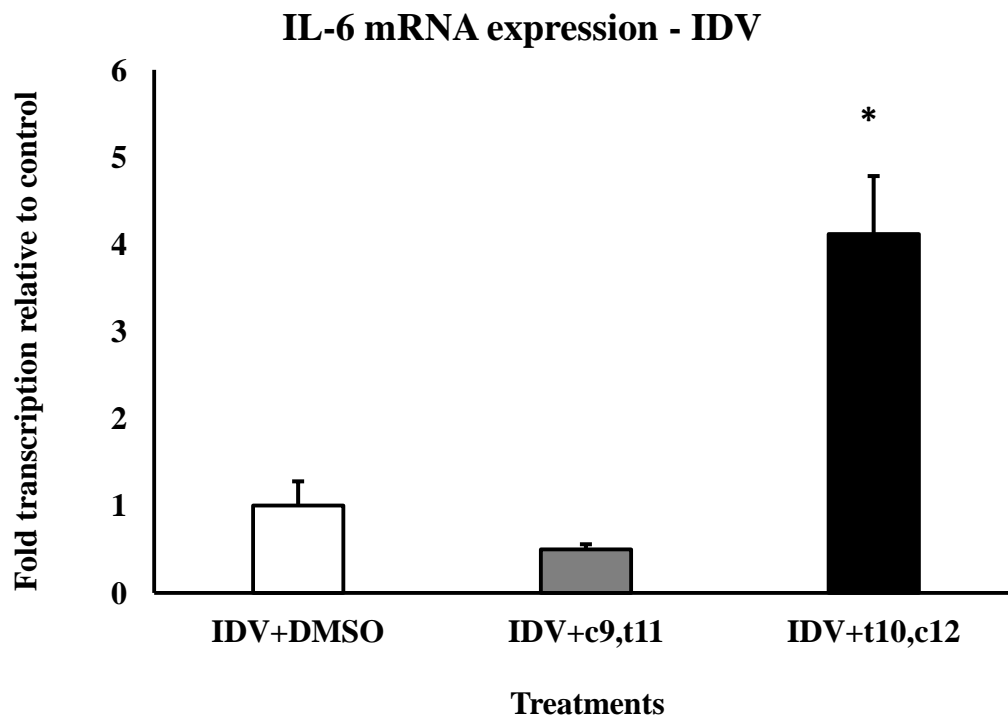


Figure 4.11 Effect of TDF and CLA on resistin transcription. Adipocytes were treated with control, near C_{\max} concentrations of TDF or 100 μM CLA isomers, c9,t11 or t10,c12. Expression of resistin mRNA was measured on day 14 relative to Hprt housekeeping gene by RT-PCR. Results represent mean \pm SEM of 4 parallel cell culture experiments. *Significantly different ($P = 0.033$) from control cells.

The effects of the two CLA isomers on IL-6 gene expression was investigated in adipocytes treated with IDV and TDF, the two ARV which significantly increased IL-6 expression in previous experiments (**Section 3.5.6**). c9,t11 had no significant effect, but t10,c12 significantly increased IL-6 expression in adipocytes treated with both IDV- and TDF-treated cells ($P = 0.001$ and $P < 0.001$, respectively; **Figure 4.12 A and B**).

A



B

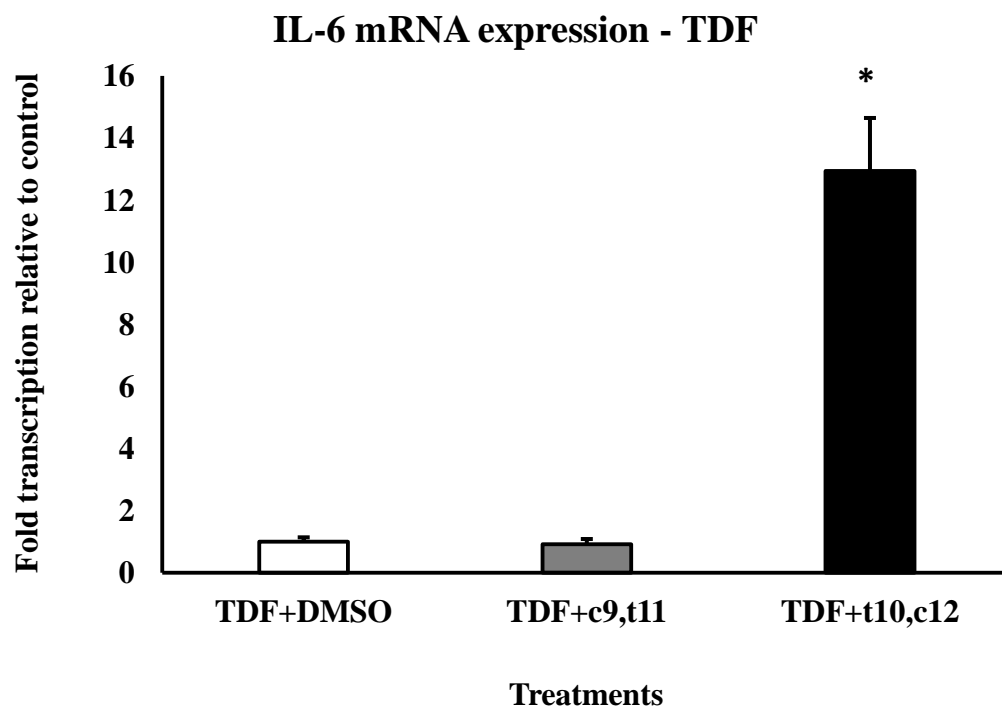


Figure 4.12 Effect of ARVs and CLA on IL-6 transcription. Adipocytes were treated with control, near C_{max} concentrations of (A) IDV or (B) TDF and 100 μ M CLA isomers, c9,t11 or t10,c12. IL-6 gene expression was measured on day 14 relative to Hprt housekeeping gene by RT-PCR. Results represent mean \pm SEM of 4 parallel cell culture experiments. *Significantly different ($P = 0.001$ and < 0.001 , respectively) from control cells.

4.5.7 Global gene expression

4.5.7.1 RTV and c9,t11

Using a threshold cut-off of 2-fold change (signal log ratio ≥ 1.0 or ≤ -1.0), only identified 3 differentially expressed genes (phospholipid scramblase 2, SRY-box containing gene 4, cDNA sequence AY036118), while detection signals for the majority of genes were close to background. Therefore, a 1.5-fold change relative to control (signal log ratio ≥ 0.5 or ≤ -0.5), was selected as a threshold to define differentially expressed genes for RTV + c9,t11 treated cells. Treatment with c9,t11 up-regulated 111 genes, while 146 genes were down-regulated compared with control. In relation to RT-PCR data, no changes ≥ 1.5 -fold were observed for PPAR- γ , perilipin, Cidea, adiponectin, leptin, resistin or IL-6 compared with control. Altered gene lists were then analysed using MetaCore[™] software (GeneGo, Thomson Reuters), which generated a list of top canonical pathway maps representing a set of signalling and metabolic maps of genes affected by RTV + c9,t11. The top 10 pathways affected by c9,t11 are listed in **Table 4.1**. No pathways were significantly altered by RTV + c9,t11. ‘Regulation of lipid metabolism by niacin and isoprenaline’ was the top pathway affected by RTV + c9,t11 in which 3 of 45 genes were altered ($P = 0.129$; FDR 0.906). **Figure 4.13** illustrates the map for this pathway. The genes up- or down-regulated within this pathway are listed in **Table 4.2**.

Table 4.1 Top 10 canonical pathways affected by RTV and c9,t11

	Pathway	<i>P</i> value	False Discovery Rate	Ratio*
1	Regulation of lipid metabolism by niacin and isoprenaline	0.13	0.91	3/45
2	Apoptosis and survival: DNA-damage-induced apoptosis	0.18	0.91	2/15
3	Beta-adrenergic-dependent CFTR expression	0.26	0.91	2/18
4	O-glycan biosynthesis	0.29	0.91	3/60
5	O-glycan biosynthesis / Human version	0.32	0.91	3/62
6	Neurophysiological process: Constitutive and regulated NMDA receptor trafficking	0.33	0.91	3/63
7	Cell cycle: Initiation of mitosis	0.14	0.91	2/25
8	Apoptosis and survival: Apoptotic Activin A signaling	0.14	0.91	2/25
9	DNA damage: ATM / ATR regulation of G2 / M checkpoint	0.15	0.91	2/26
10	Transcription: Role of Akt in hypoxia induced HIF1 activation	0.16	0.91	2/27

P value is based on the number of interactions of the gene in the pathway. FDR is calculated taking into account the *P* value for each map and the rank of the map, given the total number of maps in the whole Pathway Maps ontology. * Ratio of genes altered by RTV + c9,t11 vs. total number of genes in the pathway.

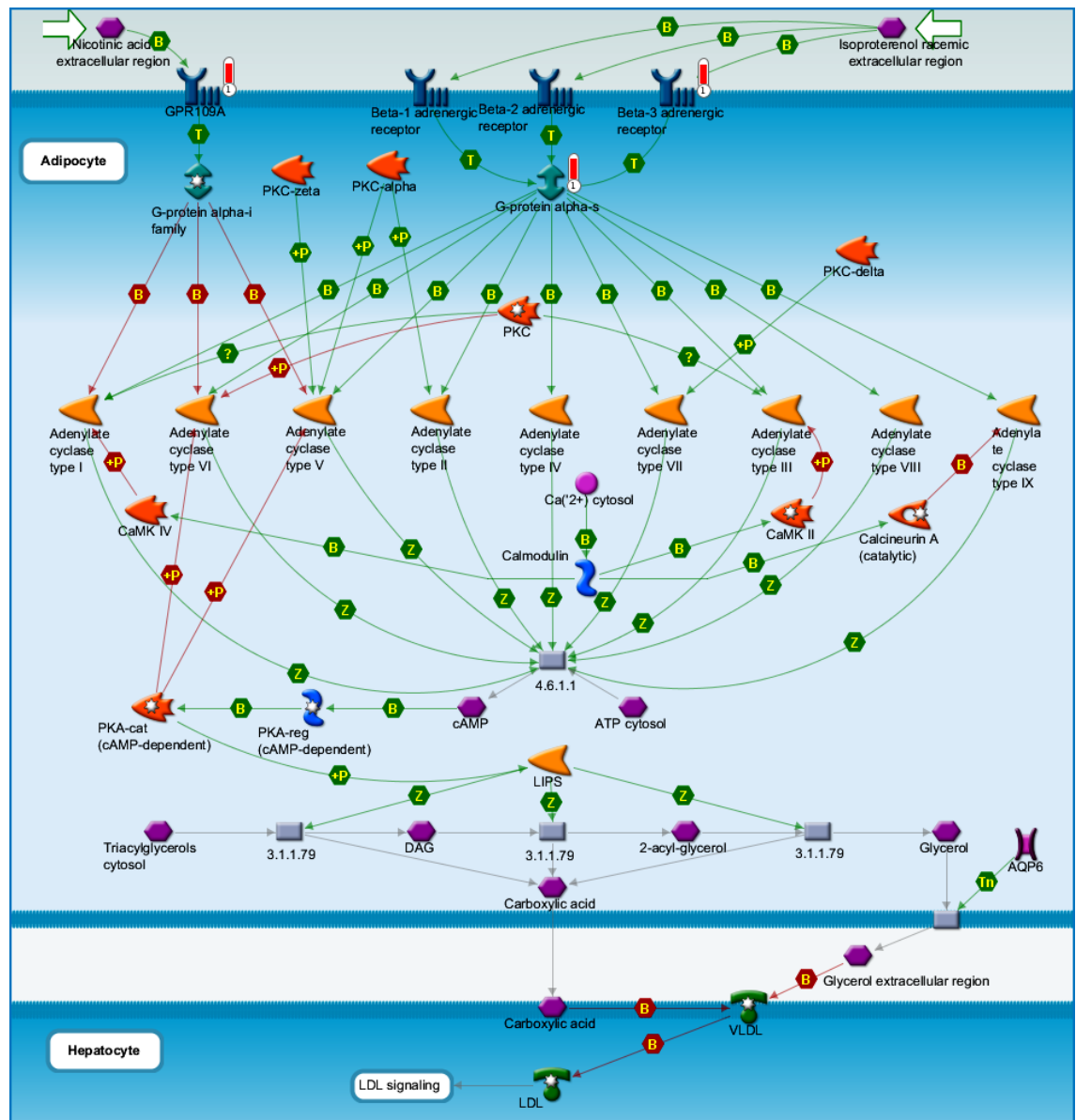


Figure 4.13 Regulation of lipid metabolism by niacin and isoprenaline (GeneGo, 2014). Histograms located next to each gene indicate the direction (blue = down-regulated; red = up-regulated) and the degree of change in expression of each gene. Full figure legend can be found in **Appendix 1**.

Table 4.2 Genes altered by RTV and c9,t11

Regulation of lipid metabolism by niacin and isoprenaline			
Gene name	Symbol	Description	Fold change*
Up-regulated			
G-protein alpha S	Gnas	Modulators of cell membrane signalling	1.6
G-protein coupled receptor	Gpr109a	Nicotinic acid receptor. Mediates increased adiponectin secretion and decreased lipolysis	1.52
Beta 3 adrenergic receptor	Adrb3	Regulates lipolysis and thermogenesis	1.5

* fold change compared with control (RTV + DMSO)

4.5.7.2 RTV and t10,c12

Using a threshold cut-off of 2-fold change (signal log ratio ≥ 1.0 or ≤ -1.0) identified 425 differentially expressed genes in RTV + t10,c12 treated cells. Treatment with t10,c12 up-regulated 165 genes, while 260 genes were down-regulated compared with control. In relation to RT-PCR data, adiponectin was down-regulated almost 3-fold and resistin down-regulated 8-fold compared with control. No change ≥ 2 -fold was observed for the other genes of interest, PPAR- γ , perilipin, Cidea, leptin or IL-6. Altered gene lists were then analysed using MetaCoreTM software (GeneGo, Thomson Reuters), which generated a list of top canonical pathway maps representing a set of signalling and metabolic maps of genes affected by RTV + t10,c12.

The top 10 pathways affected by t10,c12 are listed in **Table 4.3**. The top pathway with 10 of 52 genes altered by RTV + t10,c12 was the ‘Cell adhesion: extracellular matrix (ECM) remodelling’ pathway ($P < 0.001$; FDR = 0.008). Immune response and cholesterol biosynthesis pathways were also significantly affected. Relevant adipose tissue genes altered within the ECM remodelling and immune response pathways are presented in **Table 4.4**. Although 10 genes within the cholesterol biosynthesis pathway were altered, only two were adipose tissue genes, and as their function is not well characterised, they have not been presented here. Relevant adipocyte-specific pathways included the ‘Insulin, IGF-1 and TNF- α in brown adipocyte differentiation’ pathway in which 8 of 53 genes were altered (**Figure 4.14**; $P = 0.01$; FDR = 0.16) and the ‘Adiponectin in the pathogenesis of type 2 diabetes’ pathway in which 5 of 29 genes

were altered (**Figure 4.15**; $P = 0.03$; FDR = 0.36). The genes up- or down-regulated within these pathways are listed in more detail in **Table 4.4**.

Table 4.3 Top 10 canonical pathways affected by RTV and t10,c12

	Pathway	<i>P</i> value	False Discovery Rate	Ratio*
1	Cell adhesion: ECM remodelling	< 0.001	0.008	10/52
2	Development: Insulin, IGF-1 and TNF- α in brown adipocyte differentiation	0.01	0.16	8/53
3	Immune response: Alternative complement pathway	0.01	0.16	7/39
4	Cholesterol biosynthesis	0.01	0.09	10/103
5	Immune response: Lectin induced complement pathway	0.02	0.12	7/49
6	Expression targets of Tissue factor signalling in cancer	0.03	0.13	5/22
7	Immune response: Classical complement pathway	0.03	0.13	7/52
8	Development: Beta adrenergic receptors in brown adipocyte differentiation	0.03	0.14	6/37
9	Cell adhesion: Chemokines and adhesion	0.05	0.21	9/100
10	Adiponectin in pathogenesis of type 2 diabetes	0.03	0.36	5/29

P value is based on the number of interactions of the gene in the pathway. FDR is calculated taking into account the *P* value for each map and the rank of the map, given the total number of maps in the whole Pathway Maps ontology. * Ratio of genes altered by RTV + t10,c12 vs. total number of genes in the pathway.

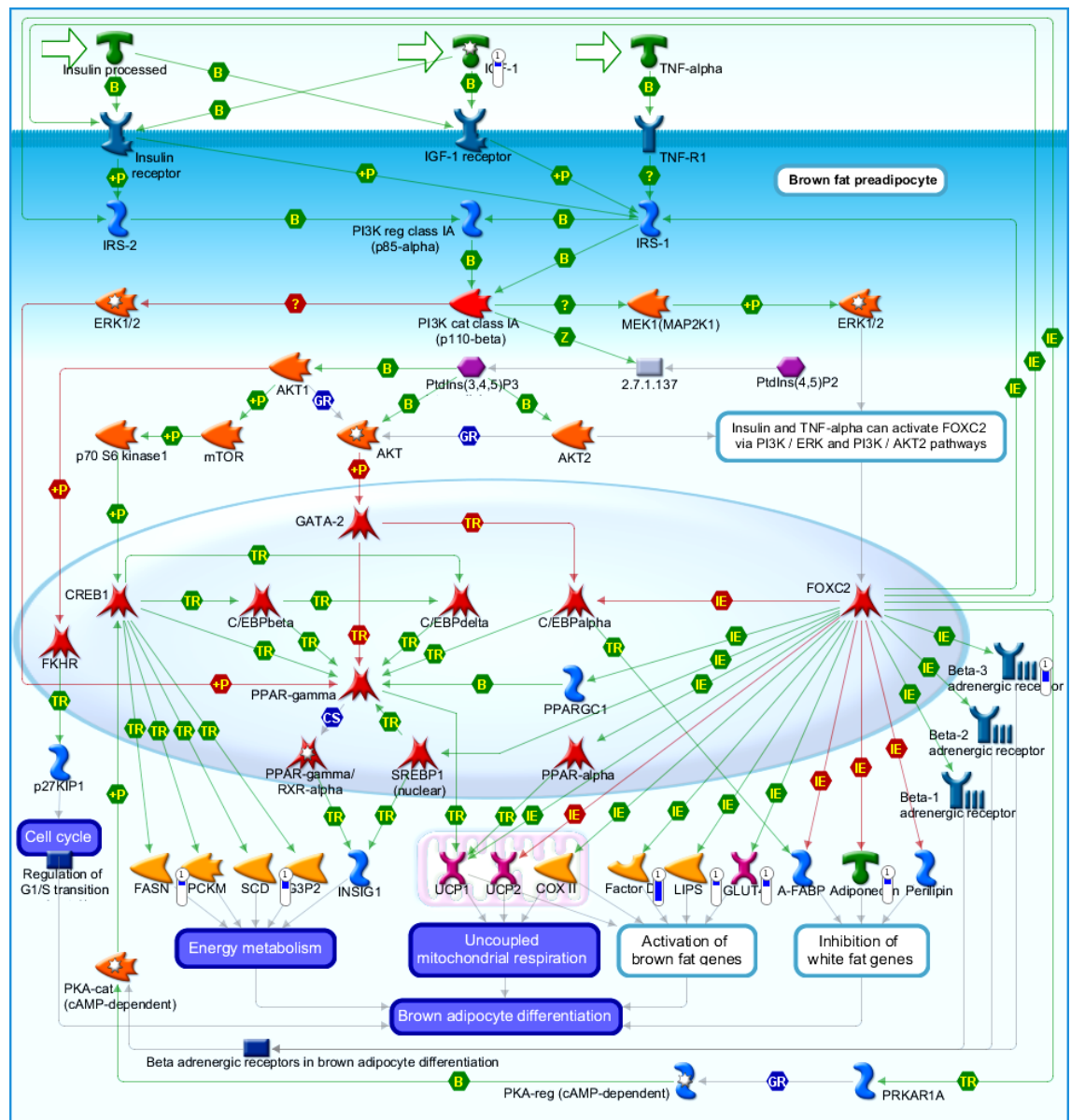


Figure 4.14 Insulin, IGF-1 and TNF- α in brown adipocyte differentiation (GeneGo, 2014). Histograms located next to each gene indicate the direction (blue = down-regulated; red = up-regulated) and the degree of change in expression of each gene. Full figure legend can be found in **Appendix 1**.

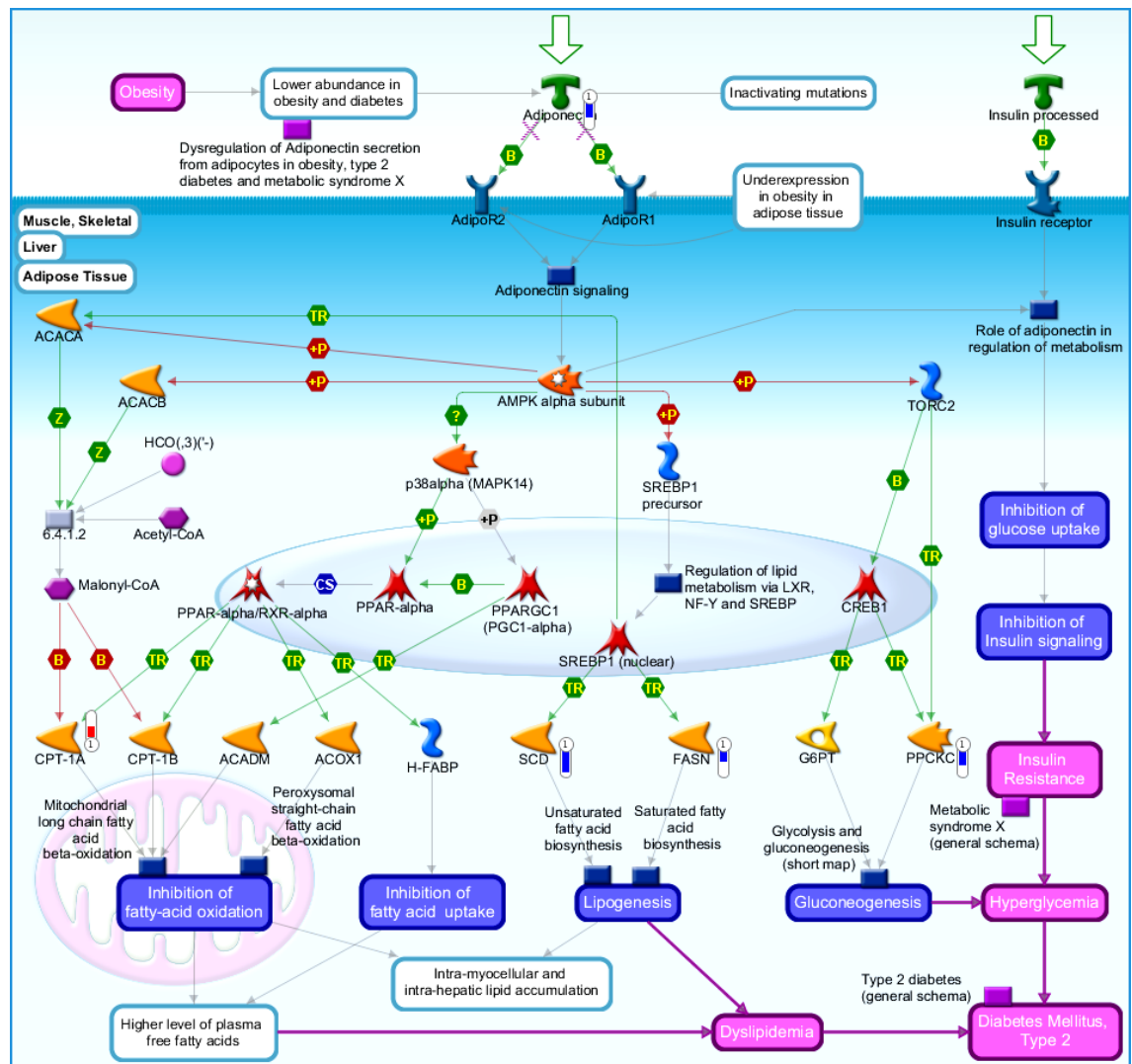


Figure 4.15 Adiponectin in the pathogenesis of type 2 diabetes (GeneGo, 2014). Histograms located next to each gene indicate the direction (blue = down-regulated; red = up-regulated) and the degree of change in expression of each gene. Full figure legend can be found in **Appendix 1**.

Table 4.4 Pathways and genes altered by RTV and t10,c12

Pathway 1: Cell adhesion: ECM remodelling			
Gene name	Symbol	Description	Fold change*
Down-regulated			
Insulin-like growth factor 1	Igf1	Involved in adipocyte differentiation and glucose metabolism	2.22
Up-regulated			
Plasminogen activator inhibitor-1	Pai1	Inhibits fibrinolysis	5.74
Metalloproteinase inhibitor 1, 2 and 3	Timp1, 2 and 3	Degradation of the extracellular matrix	2.91
Tissue-type plasminogen activator	Plat	Involved in tissue remodelling and degradation	2.63
Glia derived nexin	Serpine2	Inhibits thrombin. Binds heparin	2.55
Collagenase 3	Mmp13	Degradation of the extracellular matrix, specifically collagen	2.38
Urokinase plasminogen activator surface receptor	Plaur	Localises and promotes plasmin formation	2.34
Urokinase plasminogen activator surface receptor	Hbegf	Growth factor	2.06
Pathway 2: Insulin, IGF-1 and TNF-α in brown adipocyte differentiation			
Gene name	Symbol	Description	Fold change*
Down-regulated			
Adipsin	Cfd	Stimulates glucose transport for TG accumulation in adipocytes and inhibits lipolysis	11.21
Beta 3 adrenergic receptor	Adrb3	Regulates lipolysis and thermogenesis	6.36
Acyl CoA desaturase	Scd	Catalyses synthesis of unsaturated fatty acids	4.34
Glucose transporter type 4	Glut4	Insulin-dependent glucose transporter induced by PPAR γ	3.67
Adiponectin	Adipoq	Adipokine involved in fat metabolism and insulin sensitivity	2.95

Gene name	Symbol	Description	Fold change*
Insulin-like growth factor 1	Igf1	Involved in adipocyte differentiation and glucose metabolism	2.22
Hormone sensitive lipase	Lips	Hydrolyses stored TG	2.1
Fatty acid synthase	Fasn	Catalyses fatty acid synthesis	2.1
Pathways 3, 5, 7: Immune response: complement pathways			
Gene name	Symbol	Description	Fold change*
Down-regulated			
Adipsin	Cfd	Stimulates glucose transport for TG accumulation and inhibits lipolysis.	11.21
Complement C3	C3	Activates complement system	3.14
Up-regulated			
Complement component C1q receptor	C1qrp	Receptor for complement component 1q	3.47
Pathway 10: Adiponectin in the pathogenesis of type 2 diabetes			
Gene name	Symbol	Description	Fold change*
Down-regulated			
Stearoyl CoA desaturase	Scd	Involved in fatty acid biosynthesis, primarily oleic acid.	4.34
Adiponectin	Adipoq	Adipokine involved in fat metabolism and insulin sensitivity.	2.95
Phosphoenolpyruvate carboxykinase	Pepck	Gluconeogenic enzyme	2.9
Fatty acid synthase	Fasn	Catalyses fatty acid synthesis	2.1
Up-regulated			
Carnitine O palmitoyltransferase-1	Cpt1a	Catalyses transfer of acyl group of long-chain fatty acyl-CoA	2.19

* fold change compared with control (RTV + DMSO).

4.6 Discussion

For the first time, data presented here demonstrate significant effects of the two main CLA isomers on adipocyte function, differentiation and inflammation in adipocytes treated with ARVs. We have shown effects for CLA isomers on TG accumulation, lipid-droplet protein mRNA expression, PPAR- γ nuclear binding, adiponectin mRNA expression and protein secretion, and mRNA expression of IL-6, leptin and resistin. Microarray data identified additional genes and pathways including differentiation, lipid metabolism and immune response pathways, which were altered by RTV + CLA and may be targets for future research.

4.6.1 Effect of CLA isomers on TG accumulation

We have shown that the addition of both c9,t11 and t10,c12 to adipocytes cultured in the presence of RTV resulted in significant increases in TG accumulation compared with control. Oil Red O stained images show altered TG distribution and reduced lipid droplet size in t10,c12-treated adipocytes. Similar to our results, c9,t11 has previously been shown to increase the quantity and size of mature 3T3-L1 adipocytes as determined by ORO staining (Sakuma et al., 2010). The increase in TG accumulation observed in the presence of t10,c12 in our study contrasts with previous studies in non-RTV-treated cells where t10,c12 has been shown to decrease TG accumulation (Brown et al 2001, Evans 2001, Granlund et al 2003, Miller et al 2008). However, in another study, a mixture of the two main CLA isomers promoted lipid filling in 3T3-L1 preadipocytes (Satory and Smith, 1999). The presence of RTV in our model may have reversed the normal adipocyte response to t10,c12. A study in porcine peripheral blood mononuclear cells has shown that a combination of LPS and t10,c12 reverses the effects of the CLA isomer on NF- κ B activity and TNF- α expression (Kim et al., 2011). This suggests that t10,c12 may act differently depending on prevalent conditions.

4.6.2 Effect of CLA isomers on lipid droplet-associated proteins

Lipid droplet-associated proteins perilipin and Cidea act as a barrier, protecting TG stored within the lipid droplet from the lipolytic action of cellular lipases (Puri et al., 2008). An analysis of lipid droplet-associated proteins was undertaken in an attempt to investigate their role in the observed effects on TG accumulation. t10,c12 significantly decreased perilipin gene expression. Two studies have shown similar effects for t10,c12, but in non-RTV-treated cells only (Brown et al., 2003, Kennedy et al., 2008). Overall, previous studies investigating the effect of t10,c12 on perilipin in non-RTV-treated cells are inconsistent. In 3T3-L1 adipocytes, treatment for 8 days with 75 μ M t10,c12 increased perilipin expression 6-fold (Zhai et al., 2010). Another study in stromal vascular human adipocytes treated with 30 μ M t10,c12 showed that perilipin protein signals increased in the first 12 h, decreased at day 4 and the protein signal could not be detected at day 8 (Chung et al., 2005). These conflicting results mirror those from studies in mice where feeding 0.5% w/w CLA has been shown to increase perilipin expression (Ippagunta et al., 2011), while others showed inhibition of perilipin expression with 1% (House et al., 2005) and 1.5% mixed CLA isomer supplementation (Cai et al., 2012).

t10,c12 also significantly reduced Cidea gene expression in IDV- and TDF-treated cells in the present study. In contrast, another study showed that 0.06% w/w CLA isomer mix significantly increased Cidea mRNA expression in mice, albeit not treated with ARVs (Shen et al., 2013). Our findings for lipid droplet proteins do not corroborate those of TG accumulation and mean that an alternative mechanism underlies the increased TG accumulation in response to the CLA.

4.6.3 Effect of CLA isomers on PPAR- γ

PPAR- γ plays a crucial role in regulating adipogenesis and lipid metabolism in mature adipocytes (Desvergne and Wahli, 1999). Binding of lipophilic ligands to PPAR- γ leads to the transcription of adipocyte genes including perilipin (Dalen et al., 2004), Cidea (Puri et al., 2008) and adiponectin (Iwaki et al., 2003). In the current study, we investigated the effects of CLA isomers on PPAR- γ expression in cells treated with RTV, the only ARV to significantly affect PPAR- γ expression in previous experiments. c9,t11 increased PPAR- γ gene expression by 70%, although this did not reach statistical significance. Previous studies have shown an increase in PPAR- γ gene expression in the presence of c9,t11 in non-ARV-treated adipocytes (Belury et al., 2002, Brown et al.,

2003, Sakuma et al., 2010). t10,c12 did not significantly affect PPAR- γ gene expression in RTV-treated adipocytes. Similarly, a recent study in non-RTV treated adipocytes found no effect of DHA (natural PPAR- γ agonist) on PPAR- γ expression even though PPAR- γ target genes were reduced (Barber et al., 2013). In contrast to our findings and those of Barber *et al*, the t10,c12 isomer has previously been shown to decrease PPAR- γ expression in non-RTV-treated adipocytes (Granlund et al., 2003).

PPAR- γ nuclear binding to the PPRE was significantly increased by c9,t11 in RTV- and TDF-treated adipocytes, while t10,c12 significantly decreased PPAR- γ binding in IDV-treated adipocytes. A recent study in non-ARV-treated human umbilical vein endothelial cells found that t10,c12 at concentrations of 5, 10 and 25 μ M significantly increased PPAR- γ DNA binding activity compared with control, but 100 μ M t10,c12 had no effect (Nakamura and Omaye, 2009). In contrast, the t10,c12 isomer has previously been shown to decrease PPAR- γ mass (Miller et al., 2008), activation and protein levels (Poirier et al., 2006, Kennedy et al., 2008) in non-RTV-treated cells. At present, it appears unlikely that the increase in TG accumulation associated with t10,c12 in this study is mediated via PPAR- γ or lipid droplet proteins. In order to clarify this, an investigation of PPAR- γ target gene expression was required.

4.6.4 Effect of CLA isomers on adiponectin

Adiponectin is an insulin-sensitising adipokine induced during adipocyte differentiation (Scherer et al., 1995). PPAR- γ has been suggested to play a role in the transcriptional activation of adiponectin gene via a PPRE in the adiponectin promoter (Iwaki et al., 2003). In the present study, c9,t11 significantly increased adiponectin expression in IDV-treated cells, while t10,c12 significantly decreased its expression in RTV and TDF-treated adipocytes. A similar pattern was observed for t10,c12 in RTV- and TDF-treated cells for PPAR- γ nuclear binding, although this did not reach significance.

We subsequently investigated adiponectin secretion and found that t10,c12 significantly decreased secretion in the presence of all three ARVs. This supports RT-PCR data for the effect of t10,c12 in RTV- and TDF-treated cells, as well as the trend observed for PPAR- γ . Significant decreases in cellular and secreted adiponectin levels in response to t10,c12 have previously been shown in 3T3-L1 adipocytes in the absence of ARVs (Miller et al., 2008). Studies in mice have shown reduced adiponectin levels in response to t10,c12 treatment (Poirier et al., 2006, Miller et al., 2008). Our findings for t10,c12

are in agreement with some of those observed for PPAR- γ gene expression and PPRE binding, but also mean that the mechanism underlying the effects of t10,c12 on TG accumulation remains to be determined.

4.6.5 Effect of CLA isomers on leptin

Leptin is secreted by adipocytes in proportion to their TG stores and is thought to play a role in the regulation of energy intake and expenditure (Mantzoros, 2012). Leptin levels have been shown to be significantly reduced in HALS and are associated with insulin resistance and LA (Arama et al., 2013). In this study, c9,t11 significantly increased leptin gene expression in adipocytes treated with IDV and TDF, which corresponds with findings for TG accumulation and may explain the beneficial effect of c9,t11. Results from previous studies in non-ARV treated adipocytes are conflicting: some show that t10,c12 reduces leptin secretion in adipocytes (Kang and Pariza, 2001, Ahn et al., 2006) and serum leptin concentrations in mice (Poirier et al., 2006), while another study found no effect of either 75 μ M c9,t11 or t10,c12 on leptin mRNA expression (Zhai et al., 2010).

4.6.6 Effect of CLA isomers on resistin

In our study, t10,c12 significantly decreased resistin expression in adipocytes treated with TDF, thus ameliorating the increase in resistin expression observed in TDF-treated cells in the previous experiments (**Section 3.5.6**). Similarly, Forti and Birk (2007) demonstrated reduced resistin expression in 3T3-F442A adipocytes treated with 100 μ M CLA only; the authors do not specify which isomers were used. Resistin is an adipokine originally thought to be the link between IR and obesity (Steppan et al., 2001). Resistin expression has been shown to be regulated by PPAR- γ in 3T3-L1 adipocytes (Song et al., 2002), where over-expression of the transcription factor markedly decreases resistin expression. Some studies in mice fed 1.5% mixed CLA isomers have shown reduced resistin mRNA expression (Hernández-Díaz et al., 2010). Contrary to this, Noto *et al* (2006) showed greater resistin mRNA expression in fa/fa rats fed 1.5% mixed CLA isomers. In the context of HIV, questions exist regarding the exact role of resistin in HALS: some studies have established a link between genetic variations in resistin and metabolic complications of HAART, including IR and LD (Ranade et al., 2008, Escote et al., 2011) , while other studies failed to find any associations (Barb et al., 2005, Arama et al., 2013).

4.6.7 Effect of CLA isomers on IL-6

IL-6 has been suggested to play a role in IR by blocking insulin signalling in adipocytes (Lagathu et al., 2005). Increased IL-6 has been demonstrated in SCAT from patients with HALS (Kannisto et al., 2003) and may mediate IR in these patients. Our results show that t10,c12 significantly increases IL-6 expression in adipocytes treated with IDV and TDF, the two ARVs which significantly increased its expression in previous experiments (**Section 3.5.6**). In the absence of ARVs, t10,c12 has previously been shown to increase IL-6 expression in adipocytes (Ahn et al., 2006, Poirier et al., 2006). In contrast, c9,t11 had no effect on IL-6 expression (Ahn et al., 2006). The t10,c12-induced increase in IL-6 observed in IDV-treated cells could be suggested to contribute to the decrease in PPAR- γ binding to consensus site and adiponectin secretion observed in IDV-treated cells. Lagathu *et al* (2004) previously demonstrated that treatment of 3T3-F442A and 3T3-L1 adipocytes with IL-6 decreases PPAR- γ mRNA. Furthermore, PPAR- γ agonist rosiglitazone has been shown to decrease IL-6 (Lagathu et al., 2003). It could also be suggested that in the presence of decreased levels of PPAR- γ , IL-6 expression was no longer inhibited, leading to the expression levels we observe in IDV-treated adipocytes.

4.6.8 Global gene expression

Microarray analysis was carried out on RNA samples from adipocytes treated with a combination of RTV and either c9,t11 or t10,c12 to corroborate RT-PCR data and to identify other genes and pathways altered by RTV and CLA.

4.6.8.1 RTV and c9,t11

Microarray analysis of RNA samples from RTV and c9,t11 treated cells did not identify any changes greater than 1.5 fold in our genes of interest investigated previously using RT-PCR (PPAR- γ , perilipin, Cidea, adiponectin, leptin, resistin, IL-6). Further analysis using MetaCoreTM software (GeneGo, Thomson Reuters) identified ‘Regulation of lipid metabolism by niacin and isoprenaline’ as the top pathway altered by RTV and c9,t11, although not significantly. Isoprenaline is a β 1- and β 2-adrenoreceptor agonist and both niacin and isoprenaline bind to G-protein coupled receptor 109A (GPR109A) and affect LDL signalling. Microarray analysis revealed that GPR109A and beta 3 adrenergic receptor (ADRB3) were both up-regulated compared with control.

GPR109A is a niacin receptor, which is regulated by PPAR- γ and inhibits lipolysis by reducing cyclic AMP levels (Jeninga et al., 2009). In a previous study, acipimox, a niacin derivative and GPR109 agonist, was found to attenuate RTV-induced lipolysis in LDL receptor null mice (Guo 2009). In this study, c9,t11 appears to be acting in a similar way to niacin and niacin derivatives. Niacin has shown positive results in mitigating dyslipidaemia in patients with HALS (Dube 2006), which may occur via modulation of GPR109A expression.

Microarray analysis from **Chapter 3** revealed up-regulated ADRB3 expression in RTV-treated cells. Therefore, the addition of c9,t11 had no additional effect on the expression of the receptor and implies that RTV has potent lipolytic effects which are not easily attenuated by c9,t11. Overall, data show the potential for c9,t11 to attenuate the lipolytic effect of RTV via up-regulation of GPR109A. However, it appears to have little effect on lipolysis mediated via up-regulation of the ADRB3.

4.6.8.2 RTV and t10,c12

Adiponectin was the only gene of interest investigated using RT-PCR which was also altered in microarray analysis by RTV and t10,c12 (3-fold down-regulation). The entire altered gene list was analysed using MetaCore[™] software (GeneGo, Thomson Reuters) and a list of top 10 pathways affected by RTV and t10,c12 was generated. Similar to results from **Chapter 3**, a number of genes within the ‘Insulin, IGF-1 and TNF- α in brown adipocyte differentiation’ pathway were down-regulated compared with control, indicating that the addition of t10,c12 to RTV treated cells had no additional effect on genes expressed within this pathway.

The addition of t10,c12 to RTV treated cells altered genes within the ‘Cell adhesion: ECM remodelling pathway’, which were not previously affected by RTV alone. IGF-1 was the only gene down-regulated within this pathway and plays an important role in adipogenesis (Herrmann 2009). IGF-1 has previously been shown to be down-regulated in SCAT biopsy samples after t10,c12 supplementation (Herrmann et al., 2009). This suggests that t10,c12 might impair adipogenesis in RTV-treated cells. However, we observed an increase in TG in t10,c12 treated cells, which is therefore unlikely to involve IGF-1 expression. Plasminogen activator inhibitor-1 (PAI-1) was the top gene up-regulated within the ECM remodelling pathway. In addition to its role in ECM remodelling, PAI-1 is increasingly being investigated in relation to obesity-associated

diseases. In the context of HIV and HAART, PAI-1 was found to be reduced in RTV treated adipocytes (Mondal et al., 2001), but increased in plasma of patients with HALS (He et al., 2005). A PPRE has been identified in the PAI-1 gene promoter and PPAR- γ agonist pioglitazone has been shown to increase PAI-1 expression (Mondal et al., 2001). Although t10,c12 decreased PPAR- γ in this study, it increased PAI-1 expression similar to PPAR- γ agonists. t10,c12 has previously been shown to decrease PAI-1 expression in obese rats (Martins et al., 2010). Further studies using a PPAR- γ inhibitor are needed to investigate whether t10,c12 is mediating its effects on PAI-1 via a PPAR- γ independent mechanism.

The addition of t10,c12 to RTV treated cells altered gene expression in complement pathways involved in the immune response. Complement factor D/ adipsin (Cfd) is involved in the initial rate-limiting step of complement activation and is highly expressed in adipose tissue (White et al., 1992). CFD was down-regulated in our study. A similar finding was observed in mammary tissue of lactating mice treated with t10,c12 only (Kadegowda et al., 2013). Complement C3 (C3) was also down-regulated by RTV and t10,c12. Acylation stimulating protein (ASP) is a C3 cleavage protein and has been shown to stimulate TG storage (Paglialunga et al., 2010). Kotler *et al* (2003) found significantly lower ASP in patients with HALS compared with those without HALS and healthy controls. t10,c12 decreased both CFD and C3, and as such these two genes are unlikely to be responsible for the increase in TG storage observed in t10,c12 treated cells. However, t10,c12 may enhance the negative effects of RTV on C3, thus affecting adipogenesis. Complement component C1q receptor (C1qR) was the only component up-regulated by RTV and t10,c12. C1qR is an essential protein involved in adipogenesis and insulin signalling (Kim et al., 2009). Therefore, it could be suggested that t10,c12 may mediate some of its effects on adipogenesis via up-regulation of C1qR.

The addition of t10,c12 to RTV treated cells altered gene expression in the 'Adiponectin in the pathogenesis of type 2 diabetes' pathway. Stearoyl CoA desaturase (SCD), adiponectin, phosphoenolpyruvate carboxykinase (PEPCK) and FASN were all down-regulated by RTV and t10,c12. Microarray data support data from RT-PCR, which also showed down-regulation of adiponectin in response to RTV and t10,c12.

SCD plays an important role in *de novo* lipogenesis and its inhibition has been shown to reduce TG content in 3T3-L1 adipocytes, which is associated with down-regulation of

genes involved in TG synthesis such as glucokinase, PEPCK and PPARGC1 (Ralston et al., 2014). Down-regulation of PEPCK by t10,c12 in our study may, therefore, be explained by SCD inhibition. Although inhibition of SCD reduces adipocyte TG content, we observed an increase in TG accumulation in t10,c12 treated cells in this study, which implies that the effect of t10,c12 on TG accumulation cannot be explained by an alteration in SCD expression. FASN was down-regulated in previous experiments in **Chapter 3** by RTV and suggests that t10,c12 has no additional effect on FASN expression in RTV treated cells. Lastly, CPT1 was the only gene up-regulated in this pathway in response to RTV + t10,c12. CPT1 plays an important role in β -oxidation of fatty acids and in reducing inflammation and FFA-induced IR (Gao et al., 2011). By up-regulating CPT1, t10,c12 may be said to change adipocytes from cells that store TG to fatty acid oxidising cells. However, it also implies that the t10,c12-induced increase in TG accumulation in this study was not mediated via CPT1 expression and involves an alternative mechanism.

4.6.9 Limitations and conclusion

Limitations of our study include the fact that no combinations of ARVs were tried. Similarly, CLA isomers were also investigated separately, even though other studies have used mixed isomers and supplements for human consumption exist mainly in mixed form. In this study CLA isomers were dissolved in DMSO, rather than conjugated to bovine serum albumin, as is the normal physiological state. This may have had an effect on the bioavailability of the fatty acids. However, this study is novel and is the first to investigate the effects of CLA isomers in a model of ARV induced-adipocyte dysfunction. Overall, our results demonstrate that CLA isomers have differential effects on a number of markers of adipocyte function and differentiation. Microarray analysis identified a number of genes and pathways altered in response to RTV and CLA. Both isomers affected lipid metabolism genes and t10,c12 also affected genes involved in complement pathways. t10,c12 appears to exacerbate the effects of ARVs by down-regulating perilipin, PPAR- γ gene expression and adiponectin expression and protein secretion. In contrast, c9,t11 proved beneficial in increasing TG accumulation and nuclear activation of PPAR- γ . Although the mechanisms underlying the effects of t10,c12 induced TG accumulation remain elusive, c9,t11 may potentially mediate its effects through PPAR- γ and may, therefore, be a therapeutic option in alleviating ARV-induced down-regulation of PPAR- γ is HALS.

Chapter 5

The prevalence of lipodystrophy and factors associated with it in a London-based HIV population: data from The CREATE Study

5.1 Introduction

The use of HAART is associated with numerous toxicities including adipose tissue disturbances, which manifest as morphological and metabolic abnormalities collectively referred to as HALS (Carr et al., 1998a). While major advances have been made in elucidating the molecular mechanisms underlying HALS, a clear clinical definition for the syndrome has yet to be established. The body composition abnormalities associated with HALS are the most stigmatising and may affect quality of life and medication adherence (Shenoy et al., 2013). Additionally, metabolic abnormalities including dyslipidaemia, IR and inflammation, increase the risk of premature CVD (Galescu et al., 2013).

Due to different definitions and assessment criteria there is a wide variation in the reported prevalence of HALS. Subjective methods, such as patient self-reports and physician reports typically report prevalence figures ranging from 17% - 64%, while estimates based on objective measurement using DEXA, CT or MRI range from 10% - 83% (Loonam and Mullen, 2012). Determining its prevalence is further complicated by the fact that the terms LD and HALS are used interchangeably to define both the metabolic and morphological abnormalities associated with HIV and HAART. The distinction between LD, MetS and HALS is illustrated in **Figure 5.1**. HALS, defined as coexistence of morphological and metabolic abnormalities, has been identified by a number of groups and may have a plausible biological mechanism related to lipotoxicity and FFA uptake in VAT and non-adipose tissues (Caron-Debarle et al., 2010b). Despite this, researchers have recently begun to question the existence of HALS, suggesting the term “HALS” should be abandoned (Guaraldi and Baraboutis, 2009).

As far as could be ascertained, only one study has previously reported the prevalence of LD (defined as abnormalities of body composition only) in a UK HIV population. The Cardiovascular Risk Evaluation and Antiretroviral Therapy Effects (CREATE) Study was a cross-sectional study of HIV outpatients attending clinics in London and the surrounding area. The aim of The CREATE Study was to identify cardiovascular risk using the Framingham risk score. The prevalence of MetS in this study was 14% using NCEP criteria and 10% using IDF criteria. Median CVD risk was 4% among men and 1.4% among women as assessed using the Framingham (1991) algorithm (Aboud et al., 2010). Further analysis was conducted on a subset of the same data to determine prevalence of the MetS and factors associated with it (Elgalib et al., 2011). Elgalib *et al*

found that LD was associated with MetS defined using NCEP criteria and was prevalent in 24% of patients. As the focus of The CREATE Study was MetS and CV risk, the original researchers did not investigate the prevalence of LD in the entire cohort or the factors associated with it. Furthermore, the existence and prevalence of HALS (coexistence of body composition and metabolic abnormalities) was also not investigated.

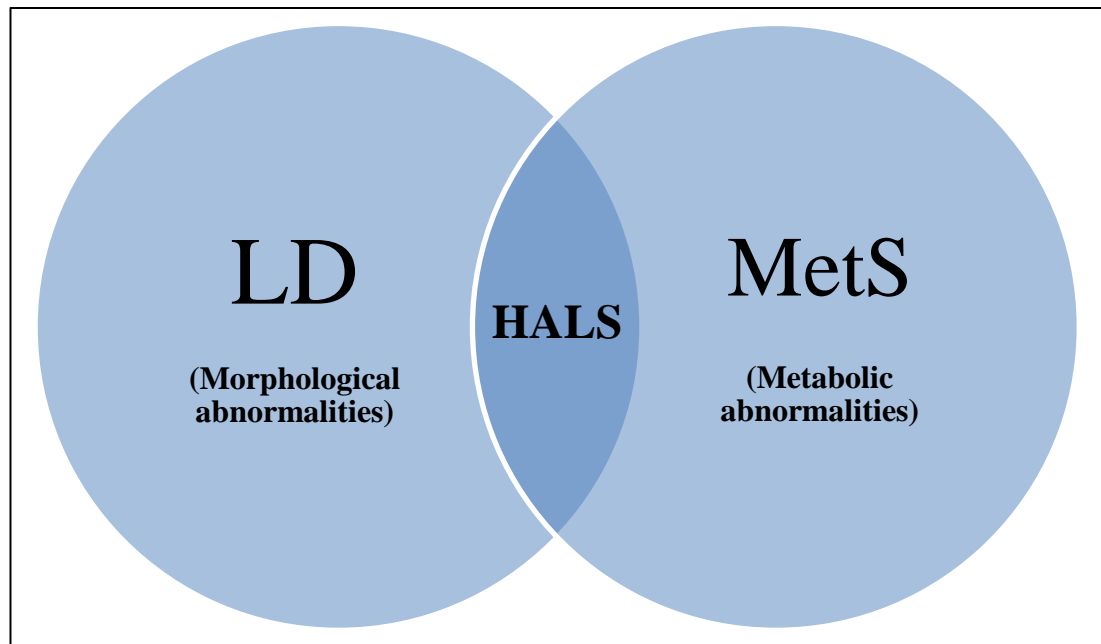


Figure 5.1 Distinction between LD, MetS and HALS

5.2 Aim

To investigate the prevalence of LD, MetS and HALS in a UK-based HIV outpatient population using data from The CREATE Study.

5.3 Objectives

1. Determine the prevalence of LD, characteristics of patients with LD and the factors associated with developing LD.
2. Determine the prevalence of the MetS and the factors associated with it.
3. Investigate whether HALS, i.e. coexistence of LD and metabolic abnormalities associated with HIV / HAART, exists in this population and if so, determine the factors associated with it.

5.4 Methods

5.4.1 The CREATE Study population and design

The CREATE Study was a cross-sectional study recruiting from among all consecutive HIV-infected individuals attending as outpatients a large inner London hospital ($n = 2500$) and a medium sized district hospital in the greater London area. Recruitment was carried out from June 2005 to September 2006 by trained research nurses and doctors. The main data on CV risk and MetS from the study has previously been published in two papers by Aboud *et al* (2010) and Elgalib *et al* (2011). Data were collected on demography, HIV disease and treatment history, current CD4 cell count and HIV viral load, as well as information on comorbidities including coinfection with hepatitis. Trained, experienced research nurses and doctors collected anthropometric data including weight, height, and waist and hip circumference according to standard protocol. LD was defined as any self- or doctor-reported change in body fat distribution in any region (LA or LH). MetS was defined using either NCEP or IDF criteria. HALS was defined as the presence of LD plus any other metabolic abnormality ($TG \geq 1.7$ mmol/L, $HDL-C < 1.03$ mmol/L for male or 1.29 mmol/L for female, $SBP \geq 130$ mm Hg, $FG \geq 5.6$ mmol/L).

5.4.2 Statistical analysis

For the present study, statistical analyses using CREATE data were performed with SPSS (version 20, IBM Corp). Chi-squared tests for categorical variables or Independent Samples t-tests for continuous variables were used to determine differences between those with and without LD. Factors thought to be associated with LD, MetS and HALS were identified *a priori* based on previous studies and were entered into a multivariable binary logistic regression model. The model for LD contained the following variables: age, gender, ethnicity, CD4 count, viral load, years with HIV, first line therapy, years on HAART, TG, TC, HDL-C, FG, SBP and WC. The MetS model contained the following: age, gender, ethnicity, CD4 count, HIV viral load, duration of HIV infection, use of first line regimen, duration of current regimen and BMI. The model for HALS contained age, gender, ethnicity, CD4 count, viral load, years with HIV, first line therapy, years on HAART and BMI. All data presented in text and tables are expressed as means \pm SD. Odds ratio (OR) and 95% confidence intervals (CI) are reported. Statistical significance was taken at $P < 0.05$.

5.5 Results

5.5.1 Patient characteristics

Baseline characteristics of patients are summarised in **Table 5.1**. The majority of patients were Caucasian (51%), homosexual (52%), male (75%) with a mean age of 40 years. Mean duration of HIV infection was 5 years and ranged from 0–26. The majority of patients had well controlled disease as indicated by a CD4 count > 300 cells/mm³ (73%) and a HIV viral load of < 50 copies/ml (62%). Three-quarters of patients were taking HAART for an average of 9 years. Of these, almost all (98%) were receiving NRTIs, while only 30% were being treated with PI. The mean TG concentration was 1.7 mmol/L, mean TC was 4.58 mmol/L and mean HDL-C was found to be 1.32 mmol/L. Mean SBP and FG were within the normal range at 120 mmHg and 5.1 mmol/L, respectively. Mean WC was below the cut-offs used to indicate risk of MetS (≥ 94 cm in men). The average BMI was 25 kg/m². When the NCEP criteria were used, 17% of patients were classified as having the MetS, which increased to 22% when using the IDF criteria. LD, defined as any body fat abnormality either self-diagnosed or diagnosed by a doctor, was present in 23% of 945 patients for whom data were available. Only a sub-set of patients ($n = 555$) had complete data for both metabolic parameters and LD. Analysis of this sub-set of data revealed that 29 % of patients had a diagnosis of HALS (the presence of LD plus any other metabolic abnormality). There was a high degree of concordance between doctor and patient diagnoses of LD as indicated by a 0.825 kappa test score ($P < 0.001$; **Table 5.2**).

Table 5.1 Patient characteristics

Characteristic	
Demographic	
Male <i>n</i> (%)	762 (75)
Female <i>n</i> (%)	260 (25)
Caucasian <i>n</i> (%)	510 (51)
African <i>n</i> (%)	334 (33)
Caribbean <i>n</i> (%)	68 (7)
Asian <i>n</i> (%)	20 (2)
Age (years)	40 ± 9
Aged > 45 <i>n</i> (%)	253 (25)
Men-sex with men <i>n</i> (%)	448 (52)
Heterosexual <i>n</i> (%)	356 (41)
Intravenous drug user <i>n</i> (%)	13 (2)
Smoker <i>n</i> (%)	378 (37)
HIV disease / drug factors	
Duration HIV infection (yrs)	5 ± 4
CD4 count, (cells/mm ³)	442 ± 222
CD4 >300 <i>n</i> (%)	736 (73)
HIV <50 copies per ml <i>n</i> (%)	623 (62)
On HAART <i>n</i> (%)	766 (75)
Duration of HAART (yrs)	9 ± 2
First line regimen <i>n</i> (%)	131 (31)
PI <i>n</i> (%)	300 (30)
NRTI <i>n</i> (%)	736 (98)
Coinfection HBV <i>n</i> (%)	48 (6)
Coinfection HCV <i>n</i> (%)	34 (4)
Metabolic / morphological	
TG (mmol/L)	1.7 ± 1.3
TC (mmol/L)	4.58 ± 1.1
HDL-C (mmol/L)	1.32 ± 0.5
SBP (mmHg)	121 ± 15
FG (mmol/L)	5.1 ± 1.3
WC (cm)	90 ± 11
Weight (kg)	76 ± 14
BMI (kg/m ²)	25 ± 5
MetS (NCEP) <i>n</i> (%)	96 (17)
MetS (IDF) <i>n</i> (%)	128 (22)
LD either diagnosis <i>n</i> (%)	216 (23)
LD diagnosed by doctor <i>n</i> (%)	178 (19)
LD diagnosed by patient <i>n</i> (%)	193 (20)
HALS <i>n</i> (%)	159 (29)

Values are mean ± SD. Data are presented for all patients who participated in the study (*n* = 1022). Data on LD were available for 945 patients, on 581 patients for MetS and on 555 patients for HALS.

Table 5.2 Concordance of doctor and patient reports of LD

		LD noticed by patient		Agreement ^a	P value
		Yes	No		
LD noticed by doctor	Yes	155	22	0.825	< 0.001
	No	29	727		

^a Kappa test for level of agreement beyond chance between doctor and patient. A score of less than 0.2 is considered poor, a score of 0.2-0.4 is fair and a score above 0.4 represents moderate-good agreement.

5.5.2 Characteristics of patients with LD compared with those without LD

Table 5.3 shows characteristics of the study sample according to the presence of LD. Patients with LD were older ($P < 0.001$), less likely to be asymptomatic ($P = 0.001$), and tended to be diagnosed with HIV for a greater length of time ($P < 0.001$) compared with those without LD. Patients with LD had higher CD4 cell counts ($P < 0.001$) and the majority had a viral load of less than 50 copies per ml compared with those without LD ($P < 0.001$). A greater proportion of patients with LD were receiving HAART ($P < 0.001$), specifically PIs ($P < 0.001$). Patients with LD were more likely to be taking their current HAART regimen for a longer duration compared with those without LD ($P = 0.001$). NRTI use was not significantly different between those with LD and those without. Coinfection with HCV was more prevalent in those with LD compared with those without LD ($P = 0.001$). Patients with LD had higher TG ($P < 0.001$), TC ($P < 0.001$) and FG ($P = 0.011$), as well as larger WC ($P = 0.003$). A greater proportion of patients with LD had MetS, defined using NCEP and IDF criteria ($P = 0.014$ and $P = 0.022$, respectively), compared with those without LD.

We further separated ARVs according to LD diagnosis (**Table 5.4**) and found that patients with LD were significantly more likely to be taking PI ($P < 0.001$), particularly RTV ($P = 0.047$). There was no significant difference between those with LD and those without in terms of the other classes of ARVs.

Table 5.3 Characteristics of patients with and without LD

	LD (n = 216)	No LD (n = 729)	P value
Demographic			
Age (years)	44 ± 9	39 ± 9	<0.001*
Gender			
Male <i>n</i> (%)	164 (76)	551 (76)	0.918
Female <i>n</i> (%)	52 (24)	178 (24)	
Smoker <i>n</i> (%)	77 (36)	273 (38)	0.700
Ethnicity			
Caucasian <i>n</i> (%)	122 (57)	358 (50)	0.104
African <i>n</i> (%)	60 (28)	244 (34)	
Caribbean <i>n</i> (%)	9 (4)	56 (8)	
Asian <i>n</i> (%)	5 (2)	12 (2)	
Risk group			
Men-sex with men <i>n</i> (%)	100 (54)	322 (52)	0.145
Heterosexual <i>n</i> (%)	66 (36)	259 (42)	
IVDU <i>n</i> (%)	4 (2)	8 (1)	
Disease factors			
Disease Stage			
Asymptomatic <i>n</i> (%)	43 (35)	265 (55)	0.001*
Symptomatic (pre-AIDS) <i>n</i> (%)	44 (36)	125 (26)	
AIDS <i>n</i> (%)	35 (29)	95 (20)	
Duration of HIV (years)	8 ± 5	4 ± 5	<0.001*
CD4 count (cells/mm ³)	517 ± 243	422 ± 212	<0.001*
HIV <50 copies per ml <i>n</i> (%)	171 (85)	375 (53)	<0.001*
Receiving HAART <i>n</i> (%)	205 (95)	496 (68)	<0.001*
First line regimen <i>n</i> (%)	17 (15)	100 (38)	<0.001*
Duration HAART (years)	9 ± 2.2	8 ± 2	0.001*
PI <i>n</i> (%)	96 (45)	180 (25)	<0.001*
NRTI <i>n</i> (%)	197 (97)	478 (98)	0.363
Hepatitis B <i>n</i> (%)	14 (8)	32 (5)	0.129
Hepatitis C <i>n</i> (%)	14 (8)	17 (3)	0.001*
Metabolic parameters			
TG (mmol/L)	2.03 ± 1.5	1.57 ± 1.0	<0.001*
TC (mmol/L)	4.98 ± 1.1	4.62 ± 1.0	<0.001*
HDL-C (mmol/L)	1.35 ± 0.5	1.31 ± 0.5	0.209
SBP (mmHg)	120 ± 14	121 ± 14	0.862
FG (mmol/L)	5.36 ± 1.7	4.95 ± 0.9	0.011*
WC (cm)	92 ± 12	89 ± 11	0.003*
BMI (kg/m ²)	25.8 ± 5	24.2 ± 5	0.574
MetS (NCEP) <i>n</i> (%)	35 (16)	74 (10)	0.014*
MetS (IDF) <i>n</i> (%)	42 (19)	96 (13)	0.022*

Values are mean ± SD. Difference between groups tested using Chi-square for categorical variables and Independent samples t-test for continuous variables. Significant difference between groups: **P* < 0.05.

Table 5.4 Antiretroviral drugs according to LD diagnosis

	LD (<i>n</i> = 216)	No LD (<i>n</i> = 729)	<i>P</i> value
PI	96 (45)	180 (25)	<0.001*
RTV	55 (27)	98 (20)	0.047*
NRTI	197 (97)	478 (98)	0.363
d4T	1 (0.5)	7 (1)	0.484
ZDV	42 (19)	131 (18)	0.623
NNRTI	108 (53)	281 (58)	0.253
NtRTI	109 (53.7)	272 (56)	0.566

Values are *n* (%). Data are reported for patients for whom data on LD diagnosis were available (*n* = 945). Significances of differences between groups determined using Chi-square test: **P* < 0.05.

5.5.3 Factors associated with LD , MetS and HALS

Factors associated with LD were investigated using multivariable binary logistic regression analysis (**Table 5.5**). The odds of having LD was 0.32 times lower for male patients (*P* = 0.023) compared with female patients and 0.21 times lower for those receiving a first line ART regimen (*P* < 0.001) compared with those not receiving a first line regimen. LD was associated with increasing years of current HAART regimen (OR 1.29, *P* = 0.007) and increased FG (OR 1.32, *P* = 0.043). Other variables included in the model, but which showed no effect, included age, ethnicity, CD4 count, HIV viral load, duration of HIV infection, BMI, WC, TC, TG, HDL-C and SBP.

MetS was significantly associated with increasing age (OR 1.05, *P* = 0.015). The odds of having MetS were greater for male patients compared with female patients (OR 2.98, *P* = 0.048). Caucasian and Asian ethnicity (OR 2.65, *P* = 0.034 and OR 7.34, *P* = 0.030, respectively) and increasing BMI (OR 1.22, *P* < 0.001) were also significantly associated with having MetS (**Table 5.6**). Factors added to the regression model, but which showed no effect include gender, CD4 count, HIV viral load, duration of HIV infection, receiving a first line regimen and duration of current HAART regimen.

Similar to LD, the odds of having HALS were reduced for those receiving a first line ART regimen compared with those not receiving a first line regimen (OR 0.28, *P* = 0.003) (**Table 5.7**). This was the only variable significantly associated with HALS in

the regression model. Variables included in the model, but which were not significant include age, ethnicity, most recent CD4 count, HIV viral load, duration of HIV infection and BMI.

Table 5.5 Association of factors with LD

Multivariable binary logistic regression			
Variable [reference]	OR	95% CI	P value
Age, years	1.02	0.98-1.06	0.401
Gender [Female]	0.32	0.12-0.86	0.023*
Ethnicity [African]			
Caribbean	0.75	0.17-3.42	0.712
Caucasian	0.87	0.37-2.03	0.747
Asian	1.04	0.10-10.5	0.977
Other	0.97	0.29-3.21	0.961
CD4 count, cells/mm ³	1.00	1.00-1.00	0.072
HIV viral load <50 [No]	1.04	0.44-2.44	0.925
Duration HIV infection, years	1.01	0.94-1.07	0.889
First line regimen [No]	0.21	0.10-0.48	<0.001*
Duration of current HAART regimen, years	1.29	1.07-1.55	0.007*
BMI, kg/m ²	0.97	0.85-1.09	0.582
WC, cm	1.02	0.97-1.08	0.467
TC, mmol/L	0.81	0.59-1.11	0.181
TG, mmol/L	1.33	0.99-1.79	0.055
HDL-C, mmol/L	1.03	0.52-2.02	0.932
FG, mmol/L	1.32	1.01-1.73	0.043*
SBP, mm Hg	0.99	0.97-1.02	0.747

Data are reported for patients for whom data on all variables were available ($n = 264$).

Table 5.6 Association of factors with MetS

Multivariable binary logistic regression			
Variable [reference]	OR	95% CI	<i>P</i> value
Age, years	1.05	1.01-1.09	0.015*
Gender [Female]	2.98	1.01-8.75	0.048*
Ethnicity [African]			
Caribbean	0.85	0.15-4.71	0.852
Caucasian	2.65	1.05-6.39	0.038*
Asian	7.34	1.19-43.2	0.031*
Other	0.99	0.21-4.71	0.988
CD4 count, cells/mm ³	1.00	0.99-1.00	0.962
HIV viral load <50 [No]	1.29	0.49-3.32	0.601
Duration HIV infection, years	1.06	0.94-1.08	0.881
First line regimen [No]	0.79	0.37-1.70	0.553
Duration of current HAART regimen, years	1.06	0.88-1.28	0.541
BMI, kg/m ²	1.22	1.13-1.31	<0.001*

MetS was defined using IDF criteria to account for the difference in WC between ethnic groups. Data are reported on patients for whom data on all variables were available (*n* = 328).

Table 5.7 Association of factors with HALS

Multivariable binary logistic regression			
Variable [reference]	OR	95% CI	P value
Age, years	1.03	0.99-1.07	0.140
Gender [Female]	0.82	0.36-2.22	0.898
Ethnicity [African]			
Caribbean	0.65	0.12-3.54	0.618
Caucasian	0.97	0.40-2.31	0.940
Asian	0.46	0.04-5.58	0.542
Other	0.79	0.23-2.77	0.712
CD4 count, cells/mm ³	1.00	1.00-1.00	0.068
HIV viral load <50 [No]	0.91	0.37-2.19	0.836
Duration HIV infection, years	1.03	0.97-1.10	0.343
First line regimen [No]	0.28	0.12-0.65	0.003*
Duration of current HAART regimen, years	1.18	0.98-1.41	0.089
BMI, kg/m ²	1.01	0.89-1.12	0.126

Data are reported for patients for whom data on all variables were available ($n = 257$).

5.6 Discussion

5.6.1 Prevalence of LD, MetS and HALS

We identified the prevalence of LD in this study as 23%. This finding is similar to (Galli et al., 2002, Puttawong, 2004) or lower than (Saint-Marc et al., 2000) that reported in previous studies. In a review of international studies on LD in HIV, we found that its prevalence ranged from 9-83% (Loonam and Mullen, 2012) due to the use of different definitions to identify the prevalence of LD. Overall, the prevalence of MetS was 17% and 22% in these patients, defined using NCEP or IDF criteria, respectively. These values are slightly lower than that reported in the general European population (24.3%) (Scuteri et al., 2014). Having identified the prevalence of LD and MetS, we decided to determine the prevalence of HALS. HALS was defined as a coexistence of LD and any other metabolic abnormality and was identified in 29% of patients. This is lower than previous cross-sectional studies using similar patient numbers (Carr et al., 1998a). Using this definition of HALS allows us to identify additional patients with HIV / HAART-associated abnormalities. This is relevant and argues against the suggestion that the definition of HALS should be abandoned.

5.6.2 Characteristics of patients with LD compared those without LD

In this study, patients diagnosed with LD were significantly older than those without LD. A greater prevalence of LD among groups of older HIV patients has also been observed by others (Bernasconi et al., 2002, Della Justina et al., 2014). Normal aging is associated with a significant increase and redistribution of adipose tissue (Zamboni et al., 2013). Fat storage in SCAT depots is reduced in aging and is associated with an increase in ectopic and VAT lipid deposition (Kuk et al., 2009). SC adipocytes appear to lose their ability to store lipid, resulting in an increase in circulating FFA (Findeisen et al., 2011). VAT then acts as a buffer for the increased circulating FFA and increases in size. This scenario of lipotoxicity also occurs in HIV patients with LD (Caron-Debarle et al., 2010b). It could therefore be suggested that the natural aging process accelerates the development of lipotoxicity and LD in HIV.

Interestingly, patients with LD had CD4 cell counts > 300 cells/mm³ and HIV viral loads < 50 copies/ ml, both of which indicate well-controlled disease. Similar findings have been demonstrated for CD4 count in a study of Rwandan patients with LA (van

Griensven et al., 2007). In contrast, another study demonstrated a greater prevalence of LA among those with lower CD4 count and higher viral load (Lichtenstein et al., 2003). HAART may play a role in the findings we observed as the majority (97.8%) of patients with well controlled disease were also receiving HAART (data not shown), which has been implicated in the development of LD. When we investigated HAART use in patients with LD compared with those without LD we found that patients with LD were more likely to be receiving HAART and for a longer period of time. Moreover, patients with LD were more likely to be receiving a PI than those without LD. ART has long been established as a cause of LD and a number of studies have found an association between increased duration of ART and the development of LD (Carr et al., 1999, Martínez et al., 2001, Berhane et al., 2012). Data presented here correspond with other studies and confirm the long-established role of ARVs in the development of LD (Carr et al., 1998a).

Coinfection with HCV is common among HIV-infected individuals (Sherman et al., 2002). In this study, patients with LD were more likely to be infected with HCV compared with those without LD. This finding is supported by data in the literature (Galli et al., 2002, Rodriguez-Guardado et al., 2003, Leclercq et al., 2013). In contrast, HCV coinfection has been found to be associated with improved lipoprotein profile (Wheeler et al., 2014) and a reduced risk of hypercholesterolaemia and hypertriglyceridaemia (Bedimo et al., 2006) and may relate to the effect of the HCV on hepatic lipoprotein metabolism and function.

Patients with LD had higher TG, TC and FG, as well as greater WC values, compared with those without LD, which is supported by data in the literature (Carr et al., 1998a, Dinges et al., 2005, Mutimura et al., 2007, Monnerat et al., 2008, Duro et al., 2013). In a recent study, high TG/high WC was found to be associated with a more harmful form of LD and increased CV risk compared with those with low TG/low WC (Janiszewski et al., 2011). This ratio may be useful in classifying patients at risk of body composition abnormalities and CVD.

Patients with LD in this study had a higher prevalence of the MetS defined using any criteria compared with those without LD. Coexistence of metabolic and morphologic abnormalities in patients with LD has been shown by a number of other studies

(Seminari, 2002, Samaras et al., 2007, Jevtovic et al., 2009, Zannou et al., 2009) and provides further evidence for the existence of HALS.

5.6.3 Factors associated with LD, MetS and HALS

This is the first study to report the factors associated with LD among UK HIV-infected patients. In this study, male gender was associated with reduced odds of LD. Increased duration of current HAART regimen was associated with increased odds of having LD, but if this was a first line regimen the odds of having LD decreased by 79%. A previous study reported no difference between males and females in terms of LD prevalence, but males tended to report peripheral LA more than females, who tended to report more central LH (Andany et al., 2011). This finding for LA and male gender has been shown by other groups (Leclercq et al., 2013). The apparent protective effect of receiving a first line regimen in our data contrasts with previous reports where a first line regimen has been associated with body composition abnormalities (Galli et al., 2002, van Griensven et al., 2007). However, in these studies, first line regimens typically contained d4T, whereas only 1 patient with LD in the CREATE cohort was receiving d4T. Newer first line regimens, consisting of drugs with fewer side-effects, may account for the lack of a negative association between first line regimen and LD in our study. The apparent protective effect of first line regimen in our study may be explained by the fact that patients only move to second line regimen if first line fails and they experience virological failure. Virological failure is associated with increased risk of MetS (Squillace et al., 2008). Patients receiving first line regimen do not have virological failure, which may affect their risk of MetS or LD.

We found that age, gender, ethnicity and BMI were significantly associated with MetS. Interestingly, no HIV disease or drug factors were significantly associated with MetS, which suggests that what we are observing in these patients is the normal MetS, as observed in the general population. A recent study from Thailand also found an association between increasing age and MetS in ART-experienced patients with HIV (Jantarapakde et al., 2014). In contrast to our findings for male gender and MetS, Jantarapakde *et al* found an association with female gender and MetS in ART-experienced patients with HIV. The association between BMI and MetS is supported by previous findings among HIV-infected patients in the literature (Jericó, 2005, Estrada et al., 2006, Alvarez et al., 2010, Alencastro et al., 2011), as well as in the general population (Scuteri et al., 2014). Similar to our study, white race has previously been

shown to be associated with the MetS (Alencastro et al., 2011). The association between MetS and Asian ethnicity in this study corresponds with findings in the general population where Asian populations typically have an increased risk of MetS, necessitating lower MetS cut-offs for early intervention to prevent T2DM and CVD (Pandit et al., 2012).

When we investigated the factors associated with HALS, we found that receiving a first line ART regimen was significantly associated with reduced odds of having HALS. No other HIV disease or drug factors were associated with HALS. Although not found in our study, previous studies report an association between HALS and increased duration of ART (≥ 1 year), d4T use and low CD4 cell count (Mauss et al., 2002, Feleke et al., 2012). Use of d4T was limited among the CREATE cohort and the majority of patients had high CD4 cell counts, which may explain the lack of associations for these parameters in our study.

Overall, the results suggest that this population presents a number of risk factors for LD than MetS. Male gender is protective against LD, but not MetS, and increased FG and BMI are associated with increased odds of having LD and MetS, respectively. HIV disease factors were not associated with any abnormality, but ART, particularly receiving a first line regimen, was found to be protective against both LD and HALS.

5.6.4 Limitations and conclusion

Limitations of this study include the fact that LD was diagnosed using subjective reports by the patient or their physician. However, as mentioned previously, this method may be the most feasible in a clinical setting where time and resources are limited. LD in this study was defined as any body fat abnormality and as a result we do not have specific information on whether body fat distribution was predominantly LA, LH or mixed. The cross-sectional design of the study does not allow for causality to be determined. However, our findings may be used for evaluating and monitoring similar HIV patient groups in an outpatient setting. Despite the limitations of the CREATE data, we found that a significant proportion of patients (almost a quarter) are affected by LD and HALS. Although use of the definition 'HALS' has been disputed, focussing on LD or MetS in isolation prevents us from seeing the entire clinical picture. Our study demonstrates that the term HALS can be used to identify patients at risk of both metabolic and morphological abnormalities associated with HIV and HAART.

Chapter 6

The effect of supplementation with conjugated linoleic acid on waist circumference in HIV-infected men receiving HAART

6.1 Introduction

Nutrition plays an important role in the management of HIV and its associated complications (American Dietetic Association, 2010). Research investigating the effects of nutrition in mitigating aspects of HALS has focused on the traditional cardiometabolic risk factors known to be responsive to diet, such as dyslipidaemia and IR. Interventions have focused on TG lowering and have found a diet low in fat (Barrios et al., 2002), particularly saturated fat (Lazzaretti et al., 2012), with a Mediterranean-style pattern to be beneficial (Tsiodras et al., 2009, Turčinov et al., 2009). Research has demonstrated a clear beneficial effect of n-3 PUFA in lowering TG, with doses ranging from 2.9 g to 6 g proven to be effective (Wohl et al., 2005, Baril et al., 2007, De Truchis et al., 2007, Gerber et al., 2008, Stradling et al., 2012). To the best of the author's knowledge, no studies have investigated the effects of CLA on any parameter in HIV-infected individuals.

Excess abdominal fat (abdominal obesity) is the main body fat abnormality observed in HIV-infected individuals receiving HAART (Loonam and Mullen, 2012). Recently, central obesity in HIV-infected individuals has been shown to be associated with neurocognitive impairment (McCutchan et al., 2012) and physical frailty (Shah et al., 2012). Abdominal obesity in HIV-infected individuals has been shown to be associated with a higher risk of heart disease compared with uninfected controls (Lake et al., 2011). Deficient lipid storage in SCAT has been suggested to result in preferential lipid accumulation in VAT, as a result of differences in inflammation, corticosteroid levels and mitochondria content between SCAT and VAT, as well as “secondary lipotoxicity”, a process whereby fatty acids released as a result of lipolysis in SCAT are taken up by VAT, leading to an increase in the size of this depot (Caron-Debarle et al., 2010b). Down-regulation of PPAR- γ as a result of ARVs (Caron et al., 2009) leading to decreased adipogenesis and increased lipolysis (Lenhard et al., 2000), has been suggested to be a driving factor in this process.

In vitro results from **Chapter 4** have shown beneficial effects of CLA in mitigating some the effects of ARVs on adipocyte metabolism. Numerous beneficial effects of CLA have been demonstrated with respect to modulation of immune and inflammatory responses (Mullen et al., 2007), regression of atherosclerosis (Toomey et al., 2006), improved lipoprotein profile (Hur et al., 2013), cancer prevention (Bhattacharya et al., 2006), bone health (Deguire et al., 2012) and obesity (Gaullier et al., 2007). The anti-

obesity effects of CLA in particular have been explored extensively. Studies in rodents have shown CLA to be beneficial in reducing adipocyte size (DeClercq et al., 2012) and inducing body fat loss (Ippagunta et al., 2011). In humans, CLA supplementation has been shown to reduce body weight (Berven et al., 2000), body fat (Blankson et al., 2000, Gaullier et al., 2005) and abdominal obesity (Riserus et al., 2001). At present, no studies have investigated the effects of CLA in an HIV population.

6.2 Hypothesis

It was hypothesised that supplementation with CLA would reduce WC, a surrogate marker of abdominal obesity and a characteristic of HALS, in HIV-infected men receiving HAART.

6.3 Study design, participants and recruitment

6.3.1 Study design

A randomised, double-blind, placebo controlled study was used to investigate the potential for CLA supplementation to reduce WC. Participants were randomly allocated to receive either CLA (3 g/d; Clarinol[®] A80) or placebo (3 g/d high oleic sunflower oil (HOSF)). The primary outcome variable was a change in WC. Secondary outcomes included BMI and percentage body fat. The study protocol was reviewed and approved by Queen Square Ethics Committee (Ref 13/LO/1145). The intervention period was 12 weeks (**Figure 6.1**) in line with previous research indicating changes in body composition were detectable after this duration of supplementation (Berven et al., 2000, Blankson et al., 2000, Risérus et al., 2002, Laso et al., 2007). Block randomisation with a block size of 10 was used to allocate patients to treatment.

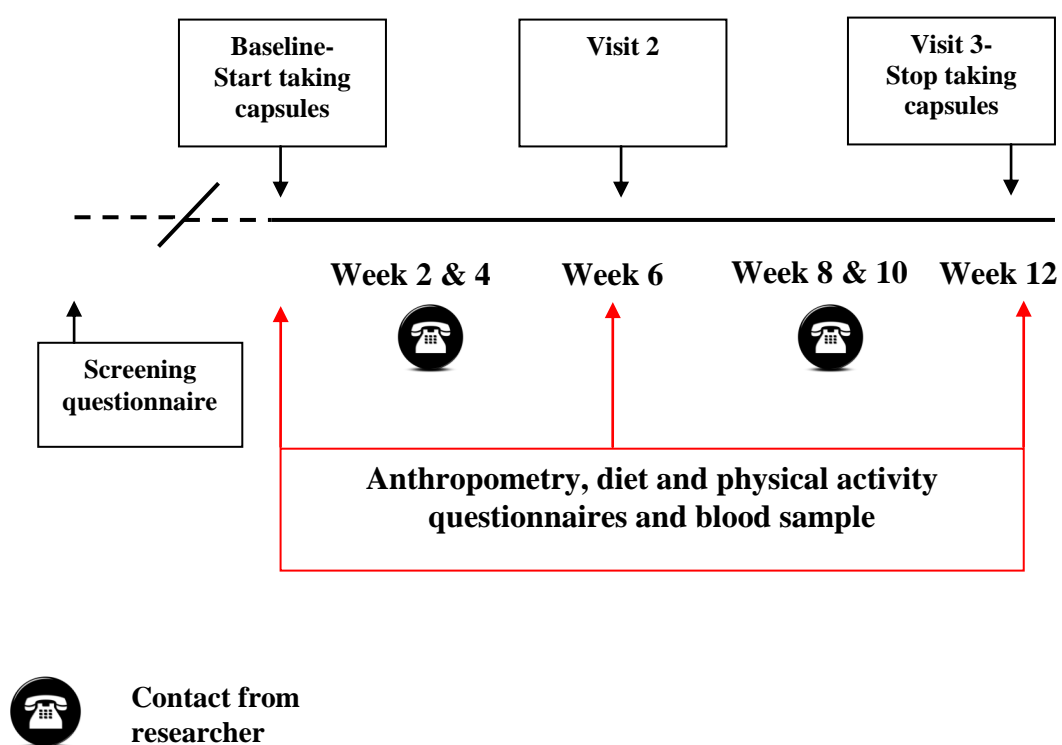


Figure 6.1 Study design

6.3.2 Experimental capsules

The experimental oils, 3 g CLA and 3 g HOSF, were supplied by Stepan Lipid Nutrition (Koog aan de Zaan, The Netherlands). CLA derived from safflower oil was provided in free fatty acid form in soft gel capsules. Each capsule contained 1 g oil, of which 78% was CLA as a 50:50 mix of c9,t11 and t10,c12. All subjects were asked to consume 4 capsules per day to provide a dose of ~3 g/d CLA. Details of the fatty acid composition of the capsules and the amount provided by 4 capsules per day can be found in **Table 6.1**. Capsules were stored in a dry, dark room at room temperature throughout the study. Capsules were packed in securitainers providing a 6-week supply (168 capsules). Patients received another 6-week supply when they returned for their mid-point visit. Patients were asked to return any remaining capsules at the 6 and 12 week visits. Unknown to the participant, 6 extra days capsules were added to each batch of supplements, which provided a measure of compliance.

Table 6.1 Fatty acid composition of the capsules and intake per day

Fatty acid	% weight		Intake (g/d)	
	Placebo	CLA	Placebo	CLA
16:0	3.86	5.22	0.15	0.21
16:1	0.11	0.09	< 0.1	< 0.1
18:0	3.06	1.84	0.12	0.07
18:1n-9	84.7	14.0	3.39	0.56
18:2n-6	7.64	0.84	0.31	0.03
18:2 cis-9,trans-11	0	39.19	0	1.56
18:2 cis,12, trans-11	0	38.80	0	1.55
20:5n-3	0	0	0	0
22:6n-3	0	0	0	0

6.3.3 Participants and recruitment

The participants were HIV-infected men recruited from the Harrison Wing, HIV outpatient clinic at St Thomas' Hospital, London. The inclusion and exclusion criteria are shown in **Table 6.2**. Patients were approached by the investigator in the clinic waiting area. The study was explained to the patient and they were asked if they would be interested in taking part. Recruitment posters and leaflets (approved by the Queen Square Ethics Committee; **Appendix 2-4**) were also displayed in the waiting area and in clinic rooms. Self-reported WC was used to determine eligibility in the waiting area and this was followed up with a screening questionnaire (**Appendix 5**) to confirm eligibility. Once eligibility was confirmed, patients were provided with an information sheet

(**Appendix 6**) and scheduled for a baseline appointment. The investigator consulted patient medical notes and electronic patient records to obtain recent blood results, as well as disease parameters and details of other relevant comorbidities which the patient was unable to provide (e.g. date of diagnosis, duration of HAART and current HAART regimen). Recruitment methodology is summarised in **Figure 6.2**.

Table 6.2 Inclusion and exclusion criteria

Inclusion criteria
<ol style="list-style-type: none"> 1. HIV-positive 2. Male 3. Aged 18 - 55 years 4. Currently receiving HAART 5. WC greater than ≥ 94 cm (or ethnic-specific cut-off) and/or BMI $> 25\text{kg/m}^2$
Exclusion criteria
<ol style="list-style-type: none"> 1. A current AIDS-defining illness 2. Any acute/chronic condition that might affect the interpretation of the results, or the participant's ability to follow protocol correctly 3. Fasting hypertriglyceridaemia (> 1.7 mmol/L) 4. Fasting hyperglycaemia (> 7 mmol/L) 5. Participants receiving testosterone replacement therapy 6. Use of any medication designed to reduce weight/ body fat 7. Participants following a diet (self- or practitioner-prescribed) to reduce weight/body fat 8. Use of fat-based supplements (e.g. fish oils) in the previous 3 months 9. Known hypersensitivity to the investigational products according to the participant and medical notes

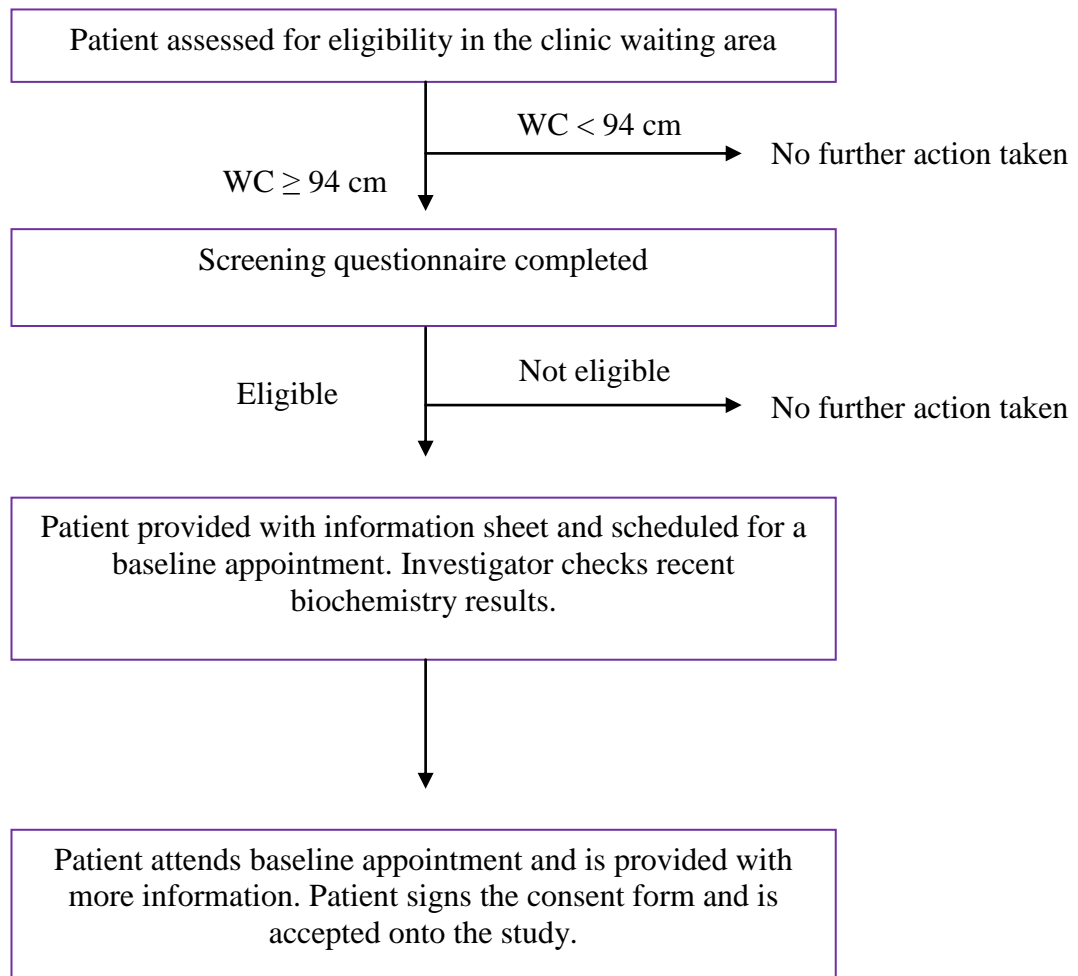


Figure 6.2 Recruitment methodology

6.4 Study visits

Eligible participants were invited to attend the HIV outpatient clinic after a 12 hour overnight fast for their first study visit, which lasted approximately 1 hour. Patients arrived between 08:30 and 11:00 hours. During this visit, the study was explained in detail and participants were given a chance to ask questions before signing a consent form (**Appendix 7**). A copy of the consent form was given to the patient and a copy placed in the medical notes along with the information sheet. A fasting blood sample was taken by a trained research nurse to assess baseline CLA levels, adipocytokines, oxidative stress, liver function, lipids, glucose and disease parameters (CD4 count and HIV viral load). In order to minimise burden to the patient, if recent results (within 3 months) were available, they were obtained from the electronic patient records and were not repeated. An anthropometric record sheet (**Appendix 8**) was completed along with a FFQ, a physical activity questionnaire and a 24 hour dietary recall. Patients were advised to take four 1 g capsules per day with or after food. At the end of the visit, a provisional 6 week appointment was scheduled and participants were provided with a monetary sum to buy breakfast before leaving the hospital. A letter was sent to the patient's GP if they requested that their GP be notified of their participation in the study (**Appendix 9**).

6.4.1 Anthropometry

More detailed instructions can be found in **Section 2.2.4**. Weight and height were measured using a digital column scale and stadiometer to the nearest 0.1 kg and 0.1 cm, respectively. WC was measured to the nearest 0.1 cm and in the standing position with arms placed on opposite shoulders, and weight evenly distributed on each leg. The measurement was taken as the midpoint between the lowest rib and the right ilium at the midaxillary line (World Health Organisation, 2008). Where possible, WC measurements were taken with the patient's waist uncovered or without heavy outer garments where this was not possible. Readings were taken at the end of a normal exhalation. Skinfolds were measured in triplicate to the nearest 0.1 mm using a Holtain calliper with the patient in standing position with arms hanging loosely by the side and weight evenly distributed on both legs. Skinfolds consisted of a double thickness of skin measured at four sites: triceps, biceps, suprailiac and subscapular. The triceps skinfold was measured on the right upper arm mid-point mark on the posterior surface. Skinfolds at the subscapular site were measured at the inferior angle of the right

scapula. Suprailiac skinfolds were measured at the high point of the right iliac crest on the midaxillary line. The average of each skinfold was calculated and as the values obtained with the calliper are not normally distributed it was necessary to log-transform the data. The total combined skinfold thickness was calculated and the resulting values used in body density equations (**Appendix 10**) to estimate percentage body fat (Durnin and Womersley, 1974). Body fat was also assessed using the Tanita TBF 300MA according to the manufacturer's instructions.

6.4.2 Dietary assessment and physical activity

More detailed instructions can be found in **Section 2.2.5-2.2.6**. The EPIC-Norfolk FFQ (**Appendix 11**) was used in this study to assess participants' usual food intake over the previous year. Participants were asked to complete an FFQ at baseline and week 12. The FFQ is a 10-page document consisting of two parts: the first and main part asks the participant to indicate their usual consumption of 130 foods from categories ranging from "never or less than once/month" to "6 times per day". Part two consists of an additional set of questions, which help to categorise breakfast cereals and gain more information about milk, total fat and fatty acid intake.

The triple pass 24-hour recall method (Nelson et al., 2008); **Appendix 12**) was used by the researcher at baseline and week 12 to assess participants' food intake during the previous 24 hours. The 24-hour recall was undertaken in chronological order of consumption, following a pattern of free and uninterrupted recall of intake, followed by detailed and specific questions about intake and quantities consumed. The recall concluded with a review of the information previously recalled, allowing for the addition of any items not remembered up to that point (Nelson et al., 2008).

A self-administered, short version of the International Physical Activity Questionnaire (International Physical Activity Questionnaire, 2001); **Appendix 13**) was used to obtain an estimate of participants' PALs during the previous 7 days. Scores for physical activity ranging from low to moderate and high were determined for each participant based on number of days and duration of vigorous and moderate activity, and walking.

6.4.3 Blood sample collection, handling and analysis

Unless results were available, venous blood samples for the analysis of plasma glucose, plasma lipids (TG, TC, HDL-C, LDL-C), liver function (albumin, alanine aminotransferase (ALT), alkaline phosphatase (ALP), bilirubin), CD4 count, HIV viral load, oxidative stress, adipocytokines and CLA levels were collected in evacuated tubes with the minimal compression necessary to display the vein. Blood for glucose was collected into 4 ml fluoride oxalate vacutainers. Blood for plasma lipids and liver function was collected into 5 ml serum tubes containing a clot activator and gel for separation. Blood for CD4 count, viral load, oxidative stress, adipocytokines and CLA levels was collected into 4 ml EDTA tubes containing anticoagulant. Tubes were then centrifuged at 2,000 rpm for 10 minutes at 4 °C and plasma samples to be used for the measurement of oxidative stress, adipocytokines and CLA were stored at -80°C until analysis. Blood samples for all other tests were sent to the respective hospital laboratory for analysis.

6.5 Determination of biochemical parameters

Determination of plasma glucose, TG, TC, HDL-C, LDL-C, liver function, CD4 count and HIV viral load was carried out by the Blood Sciences and Virology laboratories at St Thomas' Hospital. Plasma adipocytokines were measured at the Department of Clinical Biochemistry at King's College Hospital. For detailed methodology refer to **Appendix 14-22**. Oxidative stress and plasma CLA levels were determined by the investigator.

6.5.1 Determination of oxidative stress

A TBARS assay kit (Cayman Chemicals) was used to determine lipid peroxidation, an indicator of oxidative stress, in plasma samples from patients at baseline and week 12. Standards were prepared from a stock solution of 125 µM malondialdehyde (MDA) by serial dilution in the range 0 - 50 µM. 100 µl sample or standard was added to a labelled vial, to which 100 µl SDS solution was added. 4 ml of colour reagent was then added to the vial which was boiled for 1 hour. Vials were removed immediately and placed in ice water for 10 minutes. Vials were centrifuged at 2,750 rpm for 4 minutes. 150 µl of each sample was then loaded onto a colorimetric plate in duplicate and the absorbance of the solution read at 530-540 nm in a spectrophotometer (Synergy HT, Biotek). A standard curve was constructed by plotting the mean absorbance values (optical density, OD)

from the standard solutions versus the known MDA concentrations. Sample concentrations were calculated using the equation of the standard curve.

6.5.2 Determination of plasma CLA levels

The composition of CLA in plasma samples was investigated using GLC. Fatty acid methyl esters were created by transesterification as previously described (Lepage and Roy, 1986). Briefly, 100 μ l plasma was pipetted into a Teflon lined screw cap tube. 10 ml acetyl chloride was added drop-wise into 100 ml internal standard mixture. 2.2 ml of this internal standard was added to the tube. The tube was sealed and incubated overnight at 40 °C. Samples were cooled and 5 ml of 6 % potassium/sodium carbonate was added. The upper phase of the sample was collected and transferred using a Pasteur pipette to a GLC vial. 1 μ l of each sample was injected into Agilent Technologies 6890 gas chromatograph (Agilent Technologies UK Limited) fitted with a 25 m length x 0.22 mm i.d. x 0.25 μ m film thickness, Supelco SAC-5 capillary column (Sigma Aldrich) and equipped with a flame ionization detector set at 250°C. The injection was performed in split mode (50:1) with the inlet maintained at 240°C. Temperature programming was employed to optimize the separation. This was achieved by holding the initial column temperature at 160°C for 4 min, followed by ramping at 10°C/ min to 200°C and holding at this temperature for 10 min to ensure complete elution. The identities of the separated fatty acids were established by matching their retention times with those present in authentic reference standards run under identical conditions. Proportions of fatty acids as weight % were quantified by expressing the areas under the chromatographic peaks as a % of the total integrated area. Individual fatty acid contents expressed in mg/L of plasma were calculated with reference to the internal standard (15:0) peak area and the amount added to each sample. CLA isomers were identified by reference to CLA analytical standards (Sigma Aldrich).

6.6 Statistical analysis

Power calculations were conducted using WC as the primary endpoint. Using 80% power and $P < 0.05$, a formula to determine sample size was used with the aim of detecting a 5 cm difference in WC between treatments based on previous reports of SD in WC measurements of 7 cm (Kirkwood and Sterne, 2003). Therefore, the study aimed to recruit a total of 60 participants. Due to the small sample size, data were not tested for normality. Data were analysed for between subject differences from baseline to week 12 using independent samples t-tests. Paired-samples t-test was used to test for differences within the treatment groups. Statistical analysis was carried out using SPSS version 20 (IBM Corp). Statistical significance was taken at $P < 0.05$. All data presented in the text and tables are expressed as means \pm SD.

6.7 Results

6.7.1 Subjects

Recruitment onto the trial commenced in January 2014 and was completed in April 2014. The investigator assessed eligibility in 478 male patients in the HIV outpatient clinic. Of these 431 did not meet the inclusion criteria and 38 were not interested. Nine patients were eligible and expressed interest, and were provided with information and scheduled for a baseline appointment. Of these, 1 dropped out and 3 suffered adverse effects resulting in removal from the study. Details are illustrated in the consort flow chart **Figure 6.3**.

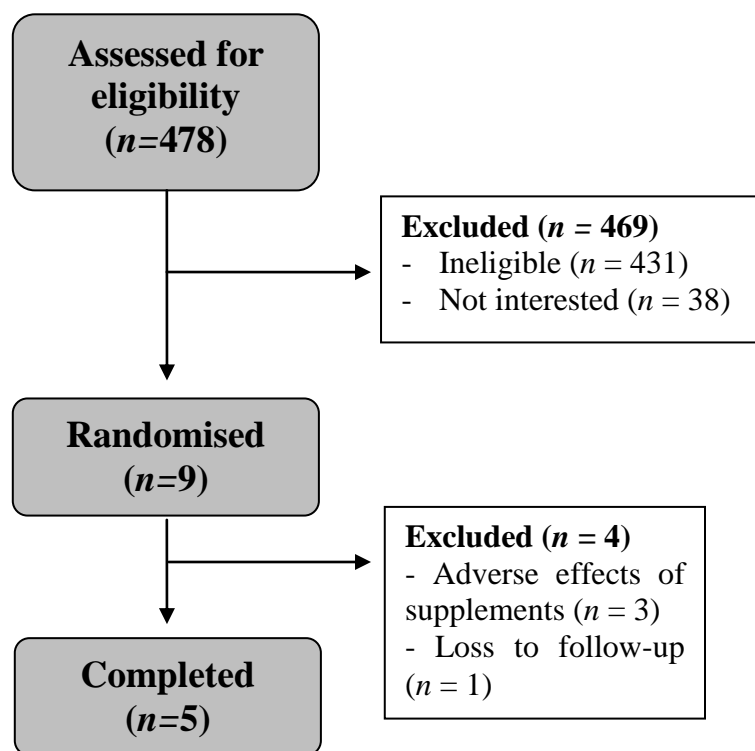


Figure 6.3 Consort flow chart

6.7.2 Baseline characteristics of subjects

A total of 5 patients completed the study. Two patients self-classified as Caucasian, two as Caribbean and one as Asian. Baseline characteristics at recruitment are presented with respect to treatment allocation in **Table 6.3**. Duration of HAART use was significantly greater among those in the CLA group at baseline ($P = 0.011$). There were no differences between the groups for any other parameter at baseline.

Table 6.3 Characteristics of subjects at baseline

Phenotype	Treatment group	
	Placebo ($n = 2$)	CLA ($n = 3$)
Age (years)	42 ± 9.9	43 ± 9.2
Years diagnosed with HIV	2.5 ± 0.7	7.3 ± 2.5
CD4 count (cells/ mm ³)	678 ± 45	623 ± 159
HIV viral load (copies/ ml) ¹	<50	<50
Years on current HAART regimen	2.5 ± 0.7	$5.7 \pm 0.6^*$
Receiving PI (n)	0	2
Receiving NRTI (n)	2	2
Height (m)	1.8 ± 0.04	1.74 ± 0.04
Weight (kg)	95.9 ± 2.7	92.7 ± 9.1
BMI (kg/m ²)	30.6 ± 2.2	30.7 ± 4.6
WC (cm)	101.6 ± 3.4	106.6 ± 2.9

Values are mean \pm SD. ¹ Values for HIV viral load for some patients were reported as “undetectable” if < 20 , so differences between groups could not be analysed and are reported as < 50 . Significant differences between the two groups using Independent-samples t-tests: $*P < 0.05$.

6.7.3 Compliance with taking the supplements

Compliance with taking the supplements was assessed by capsule counts and by measuring changes in the proportions of CLA and oleic acid in plasma. All 5 patients returned the capsules and 4 patients consumed greater than 85% of the capsules. **Table 6.4** shows plasma CLA composition (%) at baseline and after 3 g/d CLA or HOSF placebo for 12 weeks. Compared with placebo, the CLA group had significantly higher plasma 16:0 (palmitic acid; $P = 0.002$), 20:5 n-3 (eicosapentanoic acid; $P = 0.048$) and 22:6 n-3 (docosahexanoic acid; $P = 0.013$) at week 12. Within the placebo group, plasma 22:5 n-3 was lower at week 12 compared with baseline ($P = 0.015$). Plasma CLA (18:2 c9,t11 and 18:2 t10,c12) composition did not change significantly. Similarly, there was no significant change in oleic acid (18:1 n-9) after 12 weeks of treatment with HOSF capsules. Although capsule counts indicate good compliance, the proportions of c9,t11 and t10,c12 in plasma were low and did not increase significantly during supplementation as expected.

Table 6.4 Plasma fatty acid composition at baseline and week 12

	Placebo		CLA		<i>P</i> value
	Baseline	Week 12	Baseline	Week 12	
14:0	0.70 ± 0.23	0.63 ± 0.01	0.72 ± 0.06	0.94 ± 0.49	0.477
16:0	20.3 ± 0.98	20.1 ± 0.39	22.5 ± 1.38	23.5 ± 0.37	0.002*
16:1	1.32 ± 0.15	1.25 ± 0.35	1.16 ± 0.29	1.19 ± 0.35	0.855
18:0	9.03 ± 0.51	8.86 ± 0.24	9.32 ± 0.53	8.69 ± 0.47	0.692
18:1n-9	22.9 ± 5.31	24.4 ± 1.42	22.4 ± 1.34	22.0 ± 1.94	0.243
18:1n-7	1.78 ± 0.13	2.01 ± 0.07	1.52 ± 0.08	1.52 ± 0.21	0.054
18:2n-6	30.2 ± 6.90	28.62 ± 1.8	25.1 ± 3.11	25.2 ± 1.83	0.131
18:2c9,t11	0.48 ± 0.30	0.35 ± 0.14	0.41 ± 0.11	0.45 ± 0.21	0.575
18:2c12,t10	0.18 ± 0.10	0.17 ± 0.15	0.13 ± 0.06	0.18 ± 0.17	0.960
18:3n-3	0.56 ± 0.20	1.51 ± 0.84	0.77 ± 0.35	0.87 ± 0.53	0.362
18:3n-6	1.95 ± 0.35	2.11 ± 0.66	1.87 ± 0.18	1.72 ± 0.18	0.554
18:4n-3	0.56 ± 0.24	0.62 ± 0.34	0.48 ± 0.15	0.47 ± 0.10	0.653
20:3n-6	2.08 ± 0.30	1.97 ± 0.59	1.92 ± 0.24	1.72 ± 0.04	0.652
20:4n-6	4.80 ± 0.77	4.67 ± 1.21	7.89 ± 1.87	7.17 ± 1.70	0.175
20:5n-3	0.39 ± 0.22	0.48 ± 0.27	0.86 ± 0.08	1.06 ± 0.15	0.048*
22:4n-6	0.17 ± 0.01	0.13 ± 0.03	0.19 ± 0.06	0.20 ± 0.10	0.455
22:5n-6	0.16 ± 0.03	0.14 ± 0.00	0.13 ± 0.17	0.12 ± 0.11	0.075
22:5n-3	0.49 ± 0.07	0.46 ± 0.08†	0.54 ± 0.12	0.54 ± 0.12	0.457
22:6n-3	1.39 ± 0.17	1.48 ± 0.29	2.10 ± 0.39	2.45 ± 0.12	0.013*

Values are mean ± SD and expressed as % of total plasma fatty acid composition. Significant differences between the two groups using Independent-samples t-tests: * $P < 0.05$. Significant differences within groups using paired t-test: † $P < 0.05$.

6.7.4 Dietary intake and physical activity levels

Dietary intake and physical activity data are presented with respect to treatment allocation and compared within and between subjects after treatment for 12 weeks (**Table 6.5**). The total energy intake estimated using the 24 hour recall was significantly higher in the CLA group at baseline, which was due to an abnormally high intake reported by one subject on one particular day. The percentage of energy from fat was significantly higher in the CLA group at week 12 compared with placebo when dietary intake was assessed using a 24 hour recall ($P = 0.013$). Within group analysis revealed a significant decrease in energy intake from baseline to week 12 in the placebo group (37 %; $P = 0.002$). In the CLA group, the percentage of energy from total fat decreased by 27 % from baseline to week 12 ($P = 0.013$). Habitual dietary intake was assessed using the EPIC Norfolk FFQ at baseline and week 12. FFQ data revealed that energy intake was significantly lower in the CLA group compared with the placebo group at week 12 ($P = 0.028$). Within the CLA group, energy intake decreased by 50 % from baseline to week 12 ($P = 0.028$). No significant differences were observed between the groups in self-reported PALs at week 12.

Table 6.5 Dietary intake and physical activity at baseline and week 12

	Placebo		CLA		
	Baseline	Week 12	Baseline	Week 12	<i>P</i>
24 hour recall					
Energy (kcal/d)	2623 ± 172	1664 ± 176†	4014 ± 2014	1777 ± 611	0.983
Protein (% TE)	19.6 ± 2	13 ± 4.6	10.7 ± 1	20 ± 9	0.728
Fat (% TE)	31.5 ± 2	27 ± 6	50.4 ± 7	37 ± 8†	0.013*
CHO (% TE)	48 ± 4	60 ± 3	37 ± 6	39 ± 8	0.853
SFA (% TE)	9.8 ± 2	9.9 ± 6	19 ± 7	14 ± 3	0.281
MUFA (% TE)	11 ± 1	9.4 ± 3	17.6 ± 4	14 ± 2	0.340
PUFA (% TE)	7.8 ± 1	4.3 ± 2	6.8 ± 3	6 ± 2	0.782
FFQ					
Energy (kcal/d)	2046 ± 505	1558 ± 352	2515 ± 920	1261 ± 680†	0.028*
Protein (% TE)	16.8 ± 5	16 ± 1	16 ± 5	17 ± 5	0.857
CHO (% TE)	42 ± 2	48 ± 11	47 ± 8	42 ± 4	0.563
Fat (% TE)	40 ± 6	27.3 ± 6	37 ± 5	36.9 ± 8	1.000
SFA (% TE)	17 ± 2	13.7 ± 4	14 ± 6	14.4 ± 5	0.871
MUFA (% TE)	13 ± 1	11.9 ± 5	13.5 ± 2	13 ± 3	0.699
PUFA (% TE)	6.7 ± 3	5.8 ± 1	6.6 ± 3	6.8 ± 2	0.551
PAL					
Sitting (hrs/d)	8.5 ± 5	10 ± 3	9 ± 1	8 ± 4	0.591
Walking	1080 ± 711	1039 ± 0	1721 ± 2132	847 ± 480	0.628
Moderate	360 ± 509	160 ± 113	1720 ± 2200	320 ± 554	0.554
Vigorous	600 ± 849	960 ± 339	1707 ± 2956	2800 ± 4849	0.554
Total PAL	2041 ± 2069	2160 ± 226	5148 ± 4965	3967 ± 4241	0.564

TE, total energy. PAL, physical activity level. Values are means ± SD. Significant difference between treatment groups assessed using Independent-samples t-tests: **P* < 0.05. Significant difference within the same group assessed using paired t-test: †*P* < 0.05.

6.7.5 Markers of safety

Liver function, CD4 count, HIV viral load and oxidative stress were measured as markers of safety in response to the supplements (**Table 6.6**). Baseline liver function tests were not significantly different between the groups. Albumin concentration was significantly higher in the CLA group compared with placebo at week 12 ($P = 0.049$), but was still within the normal range of 35-50 g/L. CD4 cell count and HIV viral load were measured as indicators of HIV disease status. CD4 cell counts were > 300 cells/ mm^3 at baseline and did not differ significantly between the groups after 12 weeks of treatment. HIV viral load was < 50 copies per ml in all patients at baseline and was not affected by treatment with either placebo or CLA for 12 weeks. MDA is a naturally occurring product of lipid peroxidation and plasma levels were used as a marker of oxidative stress. At week 12, MDA concentration was significantly lower in the CLA group compared with the placebo group ($P = 0.002$).

Table 6.6 Markers of safety at baseline and week 12

Liver function	Reference range	Placebo		CLA		<i>P</i>
		Baseline	Week 12	Baseline	Week 12	
Albumin (g/L)	35-50	49 ± 0	46 ± 1	52 ± 2	50 ± 1	0.049*
ALP (IU/L)	30-120	71 ± 19	66 ± 12	89 ± 28	88 ± 25	0.344
ALT (IU/L)	4-59	22 ± 1	20 ± 2	31 ± 11	28 ± 13	0.445
Bilirubin ($\mu\text{mol/L}$)	3-20	5.5 ± 1	7 ± 1	8 ± 4	10 ± 9	1.000
Disease factors						
CD4 count (cells/ mm^3)	500-1500	678 ± 45	531 ± 22	623 ± 160	530 ± 154	0.998
HIV viral load (copies/ml)	20-107	< 50	< 50	< 50	< 50	-
Oxidative stress						
MDA (μM)	1.86-3.94	19 ± 8.5	11.5 ± 1.3	8.7 ± 7.4	1.4 ± 1.0	0.002*

MDA, malondialdehyde. Values are means \pm SD. Significant difference between treatment groups assessed using Independent-samples t-tests: * $P < 0.05$. Values for HIV viral load for some patients were reported as “undetectable” if < 20 , so differences between groups could not be analysed and are reported as < 50 .

6.7.6 Anthropometry

Anthropometry was assessed at baseline and week 12 in both groups using weight, WC, skinfold measurements and bioelectrical impedance analysis (**Table 6.7**). At week 12, the CLA group had significantly higher subscapular skinfold thickness compared with the placebo group ($P = 0.008$). However, it is unlikely to represent an effect of supplementation as baseline values were also significantly different. Within the CLA group, suprailiac skinfold thickness increased significantly from baseline to week 12 ($P = 0.04$).

Table 6.7 Anthropometry at baseline and week 12

	Placebo		CLA		<i>P</i>
	Baseline	Week 12	Baseline	Week 12	
Weight (kg)	95.9 ± 2.7	98.6 ± 5.1	92.7 ± 9.1	91.7 ± 8.2	0.674
BMI (kg/m ²)	30.6 ± 2.2	31.5 ± 3.2	30.7 ± 4.6	30.4 ± 4.1	0.975
WC (cm)	101.6 ± 3.4	104.5 ± 1.6	106.6 ± 2.9	106.7 ± 3.2	0.170
Tricep (mm)	14.6 ± 2.9	15.6 ± 4.4	20.6 ± 2.4	23.7 ± 3.4	0.085
Bicep (mm)	10.2 ± 7.2	12.6 ± 12.5	16.1 ± 3.4	15.3 ± 1.4	0.291
Suprailiac (mm)	24.3 ± 8.4	29.5 ± 3.6	29.6 ± 2.4	34.3 ± 3.8†	0.532
Subscapular (mm)	13.5 ± 3.7	14.5 ± 8.6	30.1 ± 2.2	28.5 ± 4.1	0.008*
Body fat- SF (%)	25.9 ± 3.9	19.4 ± 2.8	30.9 ± 3.4	33.9 ± 2.9	0.227
Body fat- BIA (%)	27.3 ± 0.14	27.4 ± 1.6	30.6 ± 3.9	30.2 ± 2.6	0.334

SF, skinfold thickness. BIA, bioelectrical impedance analysis. Values are mean ± SD. Significant difference between treatment groups assessed using Independent-samples t-tests: * $P < 0.05$. Significant difference within the same group assessed using paired t-test: † $P < 0.05$.

6.7.7 Plasma glucose and lipid concentrations

Plasma glucose and lipids were measured at baseline and after 12 weeks of supplementation with CLA or placebo (**Table 6.8**). There were no significant differences between the placebo and CLA groups at week 12. No within group differences were observed at week 12 for either group.

Table 6.8 Plasma glucose and lipid concentrations at baseline and week 12

	Placebo		CLA		<i>P</i>
	Baseline	Week 12	Baseline	Week 12	
FG (mmol/L)	5.1 ± 0.4	5.2 ± 0.5	5.1 ± 0.6	5.2 ± 0.6	0.955
TG (mmol/L)	1.4 ± 0.9	0.5 ± 2.1	1.5 ± 0.8	0.8 ± 1.6	0.564
TC (mmol/L)	4.1 ± 0.3	4.2 ± 0.8	5.1 ± 1.2	5.4 ± 1.1	0.304
HDL-C (mmol/L)	1.0 ± 0.1	1.1 ± 0.1	1.4 ± 0.2	1.5 ± 0.4	0.293
LDL-C (mmol/L)	2.4 ± 0.1	2.6 ± 0.5	2.9 ± 0.9	3.3 ± 0.9	0.439
TC:HDL-C	3.9 ± 0.6	3.8 ± 0.6	3.7 ± 1.1	3.8 ± 1.4	0.951

Values are means ± SD.

6.7.8 Plasma adipocytokine concentrations

Plasma adipocytokine concentrations were measured to determine the effect of the supplements on markers of insulin sensitivity and inflammation. **Table 6.9** shows the results at baseline and after 12 weeks of treatment. There were no significant differences between the groups at week 12 for any of the adipocytokines investigated.

Table 6.9 Plasma adipocytokine concentrations at baseline and week 12

	Placebo		CLA		<i>P</i>
	Baseline	Week 12	Baseline	Week 12	
Adiponectin	6.52 ± 1.1	6.15 ± 0.1	4.69 ± 3.3	4.96 ± 3.4	0.669
IL-2	0.86 ± 0.3	0.79 ± 0.4	0.40 ± 0.4	0.71 ± 0.2	0.819
IL-4	1.12 ± 0.3	1.15 ± 0.1	1.51 ± 0.3	1.18 ± 0.2	0.554
IL-6	1.07 ± 0.3	1.13 ± 0.1	0.71 ± 0.2	0.88 ± 0.3	0.367
IL-8	2.17 ± 0.4	2.23 ± 0.7	2.29 ± 0.3	1.65 ± 0.7	0.436
IL-10	0.55 ± 0.1	0.42 ± 0.1	0.45 ± 0.1	0.61 ± 0.6	0.564
VEGF	11.4 ± 2.9	9.38 ± 0.7	13.5 ± 4.6	9.42 ± 4.6	1.000
IFN-γ	0.25 ± 0.1	0.29 ± 0.6	0.13 ± 0.1	0.25 ± 0.2	0.852
TNF-α	2.50 ± 0.5	2.30 ± 0.6	1.92 ± 0.2	2.20 ± 0.2	0.851
IL-1a	0.16 ± 0.1	0.22 ± 0.1	0.15 ± 0.2	0.19 ± 0.1	0.813
IL-1b	0.81 ± 3.2	0.72 ± 0.3	0.29 ± 0.3	0.75 ± 0.3	0.904
MCP-1	80.1 ± 13	94.4 ± 48	112 ± 38	86.6 ± 19	0.803
EGF	6.22 ± 0.8	6.91 ± 8.2	3.03 ± 1.8	2.04 ± 1.4	0.564

Adiponectin concentration expressed as mg/L and all other cytokines as ng/L. Values are means ± SD.

6.7.9 Adverse events

All patients recruited to the study experienced adverse events, which may have been related to supplementation. The majority were gastrointestinal (GI), ranged from mild to severe in nature and included acid reflux, flatulence, soft stools, abdominal discomfort and increased urinary frequency. Three patients had to be excluded from the study due to diarrhoea. Most symptoms appeared to resolve by the 6 week visit or were mild enough for the patient to continue with the study. **Table 6.10** provides details of the adverse events according to treatment allocation. Although there was no significant difference in mean CD4 count within the groups at week 12, closer inspection of the data revealed that 4 of the 5 patients (2 placebo, 2 CLA) had lower CD4 counts at week 12 compared with baseline (**Table 6.11**).

Table 6.10 GI disturbances associated with supplementation

Adverse event	Number in placebo group	Number in CLA group	Onset	Duration	Severity (1-10)
Reflux	2	2	Day 1	6-10 days	5
Flatulence	1	0	Day 1	2 weeks	6
Increased bowel movements	0	1	Day 1	Ongoing	3
“Oiled” stools	2	1	Day 1	Ongoing	2
Soft stools	2	1	Day 7	4 weeks	3-5
Diarrhoea	1	3	Day 1	5 days	7-9
Abdominal discomfort	0	1	Day 7	4 weeks	4
Increased urination	1	0	Day 1	2-3 days	2

Table 6.11 Change in absolute and percentage CD4 counts at baseline and week 12

Patient ID	Baseline	Week 12	Treatment group
2	710 (35.53)	515 (36.77)	Placebo
3	775 (41.31)	679 (35.87)	CLA
7	638 (22.33)	372 (18.64)	CLA
8	457 (30.63)	541 (30.22)	CLA
9	646 (38.61)	547 (38.66)	Placebo

Values are CD4 count in cells/mm³ (% of total lymphocytes)

6.8 Discussion

Of 478 patients assessed for eligibility as part of this study, only 5 were eligible and completed the study. Given the small sample size it is difficult to interpret the data or to comment on the effect of CLA supplementation. The primary outcome of the study was to test whether CLA supplementation would reduce WC. No change in WC was observed after 12 weeks of CLA supplementation. The increase in EPA and DHA in the CLA group compared with placebo at week 12 may reflect an increase in dietary intake of these fatty acids, as the CLA supplements did not contain any detectable EPA or DHA.

Overall, compliance with the supplements is difficult to interpret, as plasma CLA and oleic acid composition did not increase significantly after 12 weeks of treatment. Noone *et al* (2002) have previously shown almost a two-fold increase in total plasma c9,t11 composition after 8 weeks of supplementation with 3 g/d mixed CLA isomers (0.33 g to 0.62 g / 100g total plasma lipids). Similar increases were expected in this study, but were not observed, possibly due to variability in the consumption of the supplements as a result of the adverse effects experienced by all patients. The adverse effects were considered to be probably related to the supplements, with the exception of the increased frequency of urination, which was highlighted as a previous issue and also has not been reported previously in the literature. As mentioned above, three subjects were excluded from the study due to diarrhoea, which ceased once the supplements were discontinued. The adverse effects reported in this study have been reported previously with CLA (Blankson *et al.*, 2000, Gaullier *et al.*, 2007, Onakpoya *et al.*, 2012) and HOSF supplements (Whigham *et al.*, 2004) and may relate to the gelatine capsules rather than the oil itself (EFSA, 2010)

Oxidative stress was used as a marker of safety to determine lipid peroxidation associated with treatment. MDA levels circulate in plasma at a normal concentration of 1.86-3.94 μM . In this study, baseline MDA concentrations were above the normal range in both groups. MDA has previously been shown to be increased in HIV seropositive patients compared with healthy controls and reflects disease progression (Suresh *et al.*, 2009). There was wide variability in the results from the TBARS assay carried out on these samples. This, together with the fact that such large MDA values have not been reported previously in HIV may indicate that the high levels of MDA found in this

study may be related to the experimental method. This also limits the interpretation of MDA concentrations at week 12.

The decrease in CD4 count observed in 4 of 5 patients was an unexpected finding and may be related to the supplements. However, as the effect was not treatment specific and the percentage CD4 count did not change more than 5% for any patient, it may also reflect physiological variations in this cell population. Subsequent communication with the medical team indicated that it is more likely to relate to individual variations in CD4 count or laboratory variations in its measurement.

Results from our analysis of CREATE Study data (**Chapter 4**) indicated that 25% of men had a large WC (≥ 94 cm or ethnic-specific cut-off). Based on these data, it was decided that abdominal obesity as indicated by a large WC was a problem in these patients and a measureable target for a CLA intervention study. However, over the course of recruitment for the study, it became apparent that large WC (> 94 cm or ethnic-specific cut off) was not as prevalent among the current HIV outpatient population as in 2005-2006.

It is worth noting that a relatively short timeframe was available in which to assess patients for eligibility while they were in the clinic waiting room. As a result, the initial assessment of eligibility was based on self-reported WC. It is possible that some patients' WC were greater than that reported. However, a recent study of 1,500 HIV-infected men showed that the average WC was 87 cm (95% CI 86, 87) (Falutz et al., 2014). This supports our observation that the majority of patients have a WC below the cut-off for MetS. Our findings suggest that body composition may have changed in these patients since 2005/2006. The use of newer HAART regimens with fewer lipodystrophic side-effects may account for the observed differences, or it may simply be due to a greater awareness of HALS among clinicians and patients, which leads to more timely and improved management strategies.

Notwithstanding the fact that patients did not meet eligibility criteria, a number of patients were not interested in participating. Previous studies have reported problems recruiting among this population group; Tien *et al* (2006) discontinued recruitment at two of their sites due to recruitment difficulties. During our study it was also noted that certain ethnic groups were less interested in participating in the study, a finding

observed by other groups, which has been suggested to relate to the stigma felt by these groups regarding their HIV diagnosis (Shacham et al., 2014). In the present study, other barriers to participation communicated to the researcher included patients not having the time to attend repeat study visits, not wanting to lose weight, not wanting to take extra medication and not wanting to have blood taken. Issues with transportation and time have previously been cited as barriers to recruitment among HIV-infected men (Silvestre et al., 2006). The fact that some patients chose not to participate because they did not want to lose weight is an interesting finding. Weight loss was traditionally associated with HIV wasting and was a common finding among those infected with HIV in the early years prior to HAART (Grinspoon et al., 1996). The patients who cited this reason felt that weight loss might somehow lead to disclosure of their HIV-positive status, something which they seek to conceal. This highlights the stigma that still exists around HIV and is an important consideration for studies recruiting among this patient group.

The potential therapeutic role of long chain PUFA in mitigating aspects of HALS is worth exploring in future studies. Outlined below are a number of changes which could be made in order to increase participant numbers and improve compliance:

- Using a lower WC cut-off and including other markers of the MetS such as TG, FG, HDL-C and BP.
- Recruiting patients from more than one HIV outpatient clinic in London, as well as from smaller hospitals and healthcare centres.
- The current study recruited for 4 months. Future studies should allocate more time for recruitment.
- During recruitment, it was observed that patients were more comfortable and trusting of staff with whom they were familiar and had encountered previously. Moreover, patients were more likely to participate if they were referred to the investigator by staff members. Researcher integration into the department prior to the study may increase awareness of the study and may encourage more patient referrals. This may be achieved by attending research team meetings a few months in advance of the study.
- Providing an incentive to encourage referral of patients to the study might be another useful strategy. The NIHR Clinical Research Network provides infrastructure support, including financial incentives for the Trust, for studies which meet certain criteria and are adopted on to their portfolio.

- Naturally CLA-enriched dairy products have previously been shown to be beneficial in improving lipid profiles, as well as reducing inflammation and platelet aggregation in some (Nazare et al., 2007, Pintus et al., 2013) but not all (Tricon et al., 2006, Sofi et al., 2010) studies. Future studies could explore the use of functional foods such as CLA-enriched dairy products, which may minimise the side-effects associated with oil capsules as experienced by all patients recruited to the CLAART study. Another strategy may be to use dairy products as a vehicle for the CLA, which has previously been shown to reduce body weight and total fat mass (Lopez-Plaza et al., 2013).

Overall, this study has provided useful information on HIV patients attending outpatient clinics at St Thomas' Hospital and the difficulties recruiting this group to a nutrition intervention study. Patients recruited to the study provided information on the adverse effects of supplementation and the overall feasibility of the study, which will be important to consider when planning future interventions.

Chapter 7

Final Discussion and Conclusions

7.1 Discussion

The aim of this thesis was to investigate the contribution of ARVs to the development of HALS *in vitro* and the role of CLA in mitigating adipocyte dysfunction. The prevalence of HALS and the potential role of CLA isomers in mitigating aspects of HALS in HIV-infected individuals were also investigated.

The contribution of ARVs to adipocyte dysfunction, a central mechanism involved in the pathogenesis of HALS, was investigated. RTV was found to be the most potent ARV and induced a significant decrease in TG accumulation, lipid droplet protein expression, PPAR- γ expression and nuclear consensus site binding, as well as adiponectin expression and secretion. Similar findings have been demonstrated by other groups (Lagathu, 2007, Caso et al., 2010). This is the first study to show an effect of RTV on PPAR- γ binding to its PPRE and our results suggest that RTV induces adipocyte dysfunction by inhibiting adipocyte differentiation and adipogenesis.

In line with this, microarray analysis revealed that treatment with RTV modulated the expression of master adipogenic transcription factors (CEBP- α , PPAR- γ) and adipocyte-specific markers (adiponectin, leptin, resistin, Fabp4, Cidec). The effect of RTV on PPAR- γ expression may involve altered ERK1/2 signalling or LXR- α expression, both of which could be explored in future studies using commercially available ERK inhibitors and LXR- α knockdown using RNA interference techniques.

Decreased adipocyte differentiation and adipogenesis, possibly mediated via PPAR- γ , appear to be central to adipocyte dysfunction in response to RTV treatment. However, it is also possible that increased lipolysis may play a role. Microarray analysis shows inconsistent results for lipolysis: anti-lipolysis genes such as Cidec were down-regulated, but lipolytic genes such as HSL were also down-regulated. Zinc alpha-2 glycoprotein (ZAG) is a lipid mobilising factor shown to decrease TG accumulation and increase HSL and adipose tissue TG lipase (Bao et al., 2005). The effect of ARVs on ZAG levels has not previously been investigated *in vitro*. Further investigations of ZAG will determine whether there is a role for lipolysis in ARV-induced adipocyte dysfunction.

The main limitations of this study are that no combinations of ARVs were tried, PPAR- γ agonists/ antagonists were not used and lipolysis was not investigated; thus it is important to address these points in future studies to mimic current use of fixed dose drug combinations among patients, to determine whether adipocyte dysfunction is mediated via PPAR- γ and to determine the contribution of lipid mobilising factors and lipolysis to the pathogenesis of HALS.

Due to the potent effects of ARVs on PPAR- γ , as well as the beneficial effects of PPAR- γ ligands in HALS (Raboud et al., 2010), we chose to investigate the effects of CLA isomers, as putative PPAR- γ ligands, in mitigating ARV-induced adipocyte dysfunction. Both c9,t11 and t10,c12 significantly increased TG accumulation in RTV treated cells. The reason for this increase in TG accumulation in response to t10,c12 is uncertain, but may relate to the presence of RTV in the system, which may have reversed the normal adipocyte response to t10,c12. Furthermore, the effects of t10,c12 have been shown to vary depending on the time period of treatment during adipocyte differentiation (Evans et al., 2001), which may also explain the observed effect.

The isomers had differential effects on other adipogenic and inflammatory genes. c9,t11 appears to have a beneficial effect and increased PPAR- γ binding to PPRE. In contrast, the addition of t10,c12 appears to enhance the negative effects of the ARVs by decreasing perilipin and Cidea expression, PPAR- γ binding to PPRE, adiponectin expression and secretion, as well as increasing IL-6 expression. The differential effects of CLA isomers has previously been shown (Brown et al., 2003), with t10,c12 exhibiting a more potent effect on adipocyte metabolism than c9,t11 (Zhai et al., 2010).

Microarray analysis revealed that t10,c12 was the more potent CLA isomer and significantly decreased adipogenic genes (*Igf1*, *Cfd*, *Scd*, *Adipoq*, *Pepck*, *Fasn*), which provides further evidence for role of t10,c12 in enhancing the anti-adipogenic effects of RTV. CPT-1, a key β -oxidation enzyme, was up-regulated by t10,c12 in this study and suggests that t10,c12 may increase the fatty acid oxidising capacity of adipocytes, thereby preventing TG storage and reducing fat mass. Although this contrasts with the observed increase in TG storage in t10,c12 treated cells, it highlights fatty acid oxidation as a possible area for future research.

The main limitation of this study is that no combination of CLA isomers was tried. Previous studies have used a combination of the two isomers and commercially available supplements tend to consist of an isomeric mixture. This should be addressed in future studies. The effects of the isomers could also be investigated in the presence of a PPAR- γ inhibitor such as GW9662, to determine whether the effect of t10,c12 on TG storage is mediated via a PPAR- γ -dependent mechanism.

This is the first study to report the prevalence of LD and HALS in a UK HIV population. Analysis of CREATE data revealed that 23% of patients were diagnosed with LD, up to 22% with MetS and 29% with a combination of morphological and metabolic abnormalities also known as HALS. This corresponds with previous studies, which have been found to report prevalence figures ranging from 9-83% (Loonam and Mullen, 2012).

Increased duration of HAART and raised FG levels were associated with increased odds of having LD. The associations between LD, HAART and glycaemic parameters are shown elsewhere in the literature (Lichtenstein et al., 2001, Freitas et al., 2012). Interestingly, Leclercq *et al* (2013) recently found that after 1-5 years of receiving HAART, 28% of their population developed LA; the average duration of HAART use among the CREATE cohort was 8 years.

The prevalence of MetS (up to 22%) was slightly lower in this group than in the general European population (24.3%) (Scuteri et al., 2014). The fact that no HIV disease or drug factors were associated with MetS also implies that what we observe in these patients is the normal MetS as seen in the general population.

Receiving a first line HAART regimen was protective against LD in this study, which contrasts with previous reports (Galli et al., 2002, van Griensven et al., 2007) and may indicate a yet unexplored role for virological failure in the pathogenesis of HALS.

This study is the first to identify the prevalence of LD and HALS and to determine factors associated with both in an urban UK HIV population. Findings may be useful for evaluating and monitoring similar HIV patient groups in an outpatient setting. Additionally, results show that the term HALS is useful in identifying patients with both morphological and metabolic abnormalities associated with HIV and HAART.

The main limitations of The CREATE Study pertain to data collection; no objective measure of LD was used and no details were collected regarding whether the adipose tissue alterations were peripheral (LA), central (LH) or mixed. Future studies should include objective measures of body fat distribution such as WC and BIA. Due to the problems in correctly defining HALS, future studies should also use a definition of HALS from the literature, e.g. fat mass ratio, to allow for comparison with existing data.

An intervention study was undertaken to determine the effects of CLA on abdominal obesity in HIV-infected men. Interestingly, we observed that the majority of patients attending the HIV outpatient clinic did not meet the MetS cut-off for WC. This contrasts with findings from The CREATE Study where 25% of patients attending the same clinic had a WC \geq 94 cm. The use of self-reported WC to assess eligibility in this study could be said to have underestimated the proportion of patients meeting the eligibility criteria. However, self-reported WC has previously been used to assess the prevalence of overweight and obesity (Dekkers et al., 2008) and has been found to correlate reasonably well with technician-measured WC (Rimm et al., 1990).

For those patients recruited to the study, no statistically significant change in WC was observed in the CLA group after 12 weeks of treatment, which may be due to the small sample size. However, the proportions of CLA in plasma also did not change over the course of the study, which may indicate poor compliance with supplementation. Poor compliance may relate to the GI side-effects associated with the supplements, which were experienced by patients in both treatment groups and which resulted in drop-out of three of the patients. Similar side-effects have been reported in the literature for both CLA (Blankson et al., 2000, Gaullier et al., 2007, Onakpoya et al., 2012) and HOSF supplements (Whigham et al., 2004). The fact that the side-effects were not treatment specific suggests that they may be related to the gelatine capsules, rather than to the oil itself, which has been suggested previously (EFSA, 2010).

Difficulty recruiting resulted in a small sample size and limited our ability to interpret the data. Despite this, the study provided useful information on recruiting HIV-infected men to a nutrition intervention study. Future studies should focus on amending the inclusion criteria to increase eligibility, possibly by reducing the WC cut-off or including additional MetS criteria such as TG, HDL-C, FG and BP. Another issue encountered during recruitment was that patients tended to be more trusting of staff with

whom they were familiar and therefore, more likely to engage in research if the study was introduced to them by a staff member. Future studies should be carried out over a great time period to allow the researcher to better integrate into the department and to allow sufficient time to increase awareness of the study among staff in order to encourage patient referral to the study. Incentivising recruitment within the department may also be beneficial. Future studies could also aim to recruit from more than one site, for example from smaller hospitals or through HIV charities such as The Food Chain. Less formal, more community-based settings such as these may encourage greater participation among this group for whom continuity and familiarity of care appear to be important. Lastly, a dietary intervention involving naturally CLA-enriched dairy products or dairy products as a vehicle for the CLA may be worth investigating in future studies in order to reduce the pill burden and possibly the GI side-effects associated with the supplements.

7.2 Concluding remarks and implications for future research

The strength of this study lies in the novel exploration of the effects of CLA isomers in an *in vitro* model of HALS and the translational potential of the findings among a population of HIV patients for whom observational data was available.

ARVs, particularly RTV, induced severe adipocyte dysfunction, characterised by altered differentiation and impaired adipogenesis. Future studies using a PPAR- γ inhibitor will determine whether this occurs via a PPAR- γ -dependent mechanism, and use of ERK inhibitors and LXR knockdown will identify up-stream pathways involved in PPAR- γ down-regulation. Microarray analysis identified lipolytic genes affected by RTV and suggests that lipid mobilisation may be a promising area for future research.

In preliminary data not shown here, we found that long term non-progressors (patients who maintain CD4 count > 500 cells/mm³ without ARVs) have lower concentrations of markers of adipose tissue mobilisation (ZAG) and insulin sensitivity (adiponectin) compared with those acutely infected with HIV. Recent studies have shown that ZAG concentrations in HIV positive adults on ARVs are associated with dyslipidaemia and are lower than uninfected controls (Ceperuelo Mallafré et al., 2012). Adiponectin concentrations have also been shown to be lower in HIV than in uninfected controls and are associated with severity of subclinical atherosclerosis (Ketlogetswe et al., 2014). In a recent study, higher plasma LPS, a marker of endotoxaemia, was found in patients

with HALS compared with those without HALS (Vilades et al., 2014). Future studies will investigate the effect of LPS and endotoxaemia on adipose tissue mobilisation (ZAG) and insulin sensitivity (adiponectin) as another potential mechanism underlying HALS.

CLA isomers demonstrate potential in mitigating ARV-induced adipocyte dysfunction, but the isomers have differential effects; c9,t11 may be beneficial, while t10,c12 appears to enhance the detrimental effects of ARVs. Future work should investigate the effect of a combination of CLA isomers and determine the mechanism underlying the increase in TG accumulation in response to t10,c12 using a PPAR- γ inhibitor.

A quarter of patients from the CREATE cohort were affected by HALS. As we approach ten years since The CREATE Study was conducted, it would appear timely to repeat the study to determine the current scenario of HALS in this population. Future studies should objectively measure HALS and include a definition of HALS to allow for a more robust comparison with existing literature.

The feasibility of a nutrition intervention in HIV-infected men has been explored and the barriers to recruitment and participation identified. Future randomised controlled trials investigating the effects of long chain PUFA supplementation on body fat in HIV-infected individuals are warranted, taking into account the lessons learned from the current study.

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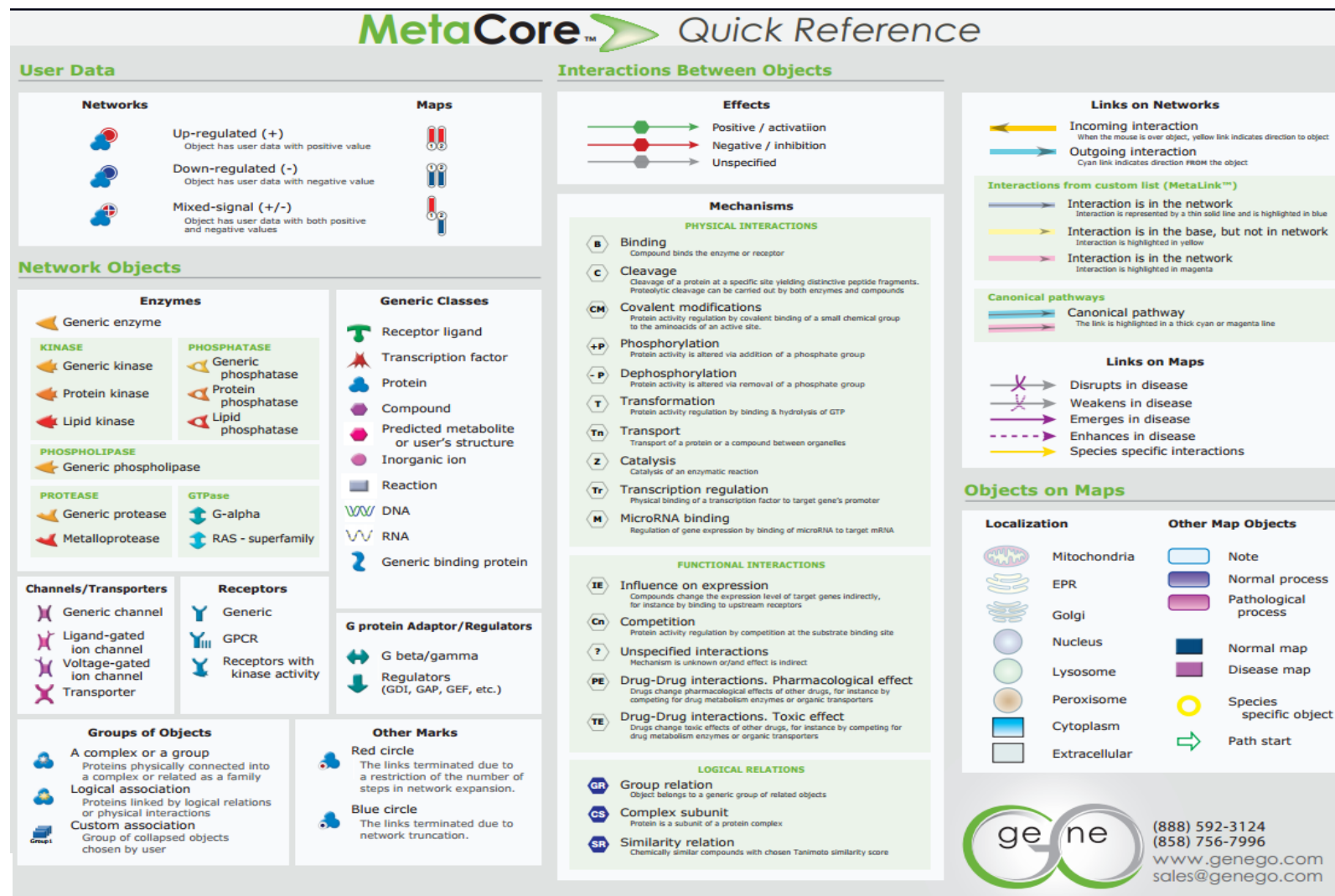
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Appendices

Appendix 1 MetaCore™ Pathway Legend



Appendix 2 Recruitment poster for patients in the waiting area

The CLAART Study Version 2 (29/01/14)

Guy's and St Thomas'
NHS Foundation Trust **NHS**

The CLAART Study

Nutrition Research



KING'S
College
LONDON

If you are

- **Male, aged between 18-55 years**
- **Receiving HAART**
- **Think your waist circumference is greater than 94 cm (37 inches) or your body mass index (BMI) is greater than 25kg/m²**

you may be able to help us with our research

We are investigating the effects of **conjugated linoleic acid**, a fatty acid found in dairy products, on levels of body fat in men receiving HAART


Eligible participants will:

- Have blood fats, glucose and liver function measured
- Have % body fat and body fat distribution measured
- Have dietary intake and physical activity assessed by a dietitian
- Receive an honorarium for time plus travel costs


If you are interested in participating in this study and would like more information, please contact Cathriona Loonam on 020 7848 4162 or by email at claartstudy@kcl.ac.uk

Appendix 3 Recruitment leaflet for patients

The CLAART Study Version 2 (29/01/14)





If you are interested in participating in this study and would like more information, contact **Cathriona Loonam** on **020 7848 4162** or by email at **claartstudy@kcl.ac.uk**

Guy's and St Thomas'
NHS Foundation Trust 

KING'S
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**NUTRITION RESEARCH
THE CLAART STUDY**

King's College London
Diabetes & Nutritional
Sciences Division,
150 Stamford Street,
London, SE19NH


**Nutrition
Research**

**The CLAART
Study**

Can you help us?

(Front and back)

The CLAART Study Version 1 (10/07/13)



The CLAART Study

You may be able to help us with our research if you are...

- Male
- Aged between 18-55 years
- Receiving HAART
- Think you may have a waist circumference greater than 94cm (37inches) or a body mass index (BMI) greater than 25kg/m²

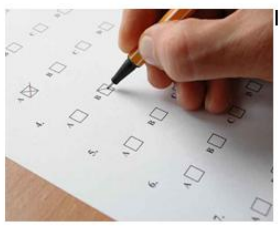


Background

Excess fat around the abdomen is one of the side-effects of HAART.

Studies have shown that conjugated linoleic acid, a fatty acid found in dairy products, may reduce body fat.

We are investigating the effects of conjugated linoleic acid on levels of body fat in people receiving HAART in the CLAART study



Eligible participants will:

- Have blood fats, glucose and liver function measured
- Have body fat distribution measured
- Have dietary intake and physical activity levels assessed by a dietitian
- Receive an honorarium for time plus travel costs

(Inside)

Appendix 4 Recruitment poster for clinic rooms

The CLAART Study Version 2 (29/01/14)

Guy's and St Thomas'
NHS Foundation Trust **NHS**

The CLAART Study

Nutrition Research



KING'S
College
LONDON

If your patient

- **is male, aged between 18-55 years**
- **receives HAART**
- **has abdominal obesity (waist circumference $\geq 94\text{cm}$) and/or a BMI $> 25\text{kg/m}^2$**

you may be able to help us with our research

We are investigating the effects of **conjugated linoleic acid (CLA)**, a fatty acid found in dairy products, on body fat composition in men receiving HAART

- Eligible participants will receive 3 g of CLA or placebo/day for 12 weeks
- Measures include: weight, height, waist circumference, body fat composition, dietary intake, physical activity levels, triglycerides, total cholesterol, glucose, liver function, oxidative stress and CLA levels
- Participants will receive an honorarium for their time plus travel costs

If you think your patient would be interested in participating please ask them to contact Cathriona Loonam on 020 7848 4162 or by email at claartstudy@kcl.ac.uk for more information

Appendix 5 Screening questionnaire

Screening questionnaire

Date _____

Name (s) _____

Address _____

Hospital no _____

D.O.B.: _____ Age: _____

Contact number/ email: _____

Receiving HAART Yes / No (circle as appropriate)

HAART regimen _____

Weight (kg) _____ or _____ (stones & lbs)

Height (m) _____ or _____ (feet & inches)

BMI (kg/m²) _____

Waist circumference (cm) _____ or (inches) _____

Are you suffering from any **acute conditions** e.g. chest infection? Yes/ No

Are you suffering from any **chronic conditions** e.g. type 2 diabetes? Yes/ No

Are you suffering from high **cholesterol**? Yes/ No

Are you suffering from high **blood glucose/sugar**? Yes/ No

Are you currently taking any **testosterone replacement therapy**? Yes/ No

Are you currently taking any **medication designed to reduce weight/**
body fat? Yes/ No

Are you currently following a **diet** (self- or practitioner-prescribed)
to reduce weight/body fat? Yes/ No

Are you currently taking or have you taken any **fat-based supplements**
(e.g. fish oils) in the previous 3 months? Yes/ No

Are you participating in any other studies? Yes/ No

Eligible for CLAART study? Yes/ No

Baseline appointment scheduled for _____

Year HIV diagnosed: _____

When HAART started: _____

Changes in regimen since then: _____

Biochemistry results

Test	Patient result	Reference range
Total cholesterol		
Triglyceride		
HDL-C		
LDL-C		
Fasting glucose		
GFR		
Albumin		
AST		
ALT		
Bilirubin		
CD4 %		
CD4		
CD4:CD8 ratio		
HIV viral load		
Hep C		
Hep B		
Other relevant		



INFORMATION SHEET FOR PARTICIPANTS

REC Reference Number 13/LO/1145

YOU WILL BE GIVEN A COPY OF THIS INFORMATION SHEET

A pilot study to investigate the effects of a novel dairy fat (conjugated linoleic acid; CLA) on body fat in men with HIV receiving HAART: the CLAART study

What is the purpose of this study?

Excess fat around the abdomen is a side-effect of highly active antiretroviral therapy (HAART). Excess fat around the abdomen increases the risk for heart disease. Studies have shown that conjugated linoleic acid (CLA), a fatty acid found in dairy products, may reduce body fat. CLA is naturally found in meat and dairy products. CLA is not produced by the human body and can only be obtained through the diet or through supplements. CLA is thought to affect body fat by altering levels of a molecule called PPAR gamma, which is known as the 'master regulator' of fat. HAART has been shown in some laboratory experiments to decrease levels of this molecule in fat cells, which may explain the loss of fat in the face, arms and legs, and increased fat around the waist sometimes seen when HAART is taken. This controlled study will investigate the effect of CLA supplements on body fat including waist circumference, with the aim of reducing fat around the waist.

We would like to invite you to participate in this original research project which is being carried out as part of the researcher's PhD project. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask me if there is anything that is not clear or if you would like more information.

Are you suitable for this study?

You have been given this information sheet as you have expressed an interest in our research. In order to participate in this study you need to be able to say 'Yes' to all of the following:

- I am male
- I am aged between 18 and 55 years
- I am receiving HAART
- I have or think I may have a waist circumference of greater than 94 cm (37 inches) or a body mass index (a measure of body fat based on your height and weight; BMI) of greater than 25 kg/m²
- I have never had a heart attack, stroke, chest pain, blood clots, heart disease, liver or kidney diseases, type 1 or type 2 diabetes, long term stomach or intestinal problems, hepatitis C or cancer
- I do not consume excess alcohol (excess alcohol = greater than 21 units per week) or use illicit drugs
- I am not taking weight loss medication or following a weight loss diet
- I am prepared to avoid consumption of fat-based supplements (e.g. fish oils) before and during the study

What will happen to you if you take part?

After completing a screening questionnaire over the telephone, if you are eligible, you will be invited to attend the Harrison Wing, St Thomas' Hospital for a meeting that should last about 1 hour. During this first visit, I will explain the study to you in detail and I will answer any questions you have.

If you would like to participate in the study, we will ask you to sign a consent form. Following consent, the following measures will be taken:

- Height using a portable height measure
- Weight using a weighing scales
- Waist circumference using a measuring tape
- Body fat (electronically using a body composition analyser and will be confirmed manually by measuring skinfold thickness at three sites: tricep, bicep, below the shoulder blade and at the top of the hip bone, using a device specially designed for this purpose)

At this stage, if your waist circumference is greater than 94 cm and/or your BMI is greater than 25 kg/m²:

- A teaspoon (5 ml) of blood will be taken by a nurse or a trained phlebotomist and used to determine levels of fat and glucose in your blood, as well as levels of free radicals (free radicals are molecules cause damage to cells in the body), liver function, immune function (CD4 count and viral load) and CLA levels.
- You will be asked to complete a food frequency questionnaire to provide information on your usual food intake
- You will be asked to complete a physical activity questionnaire to provide information on your activity levels.

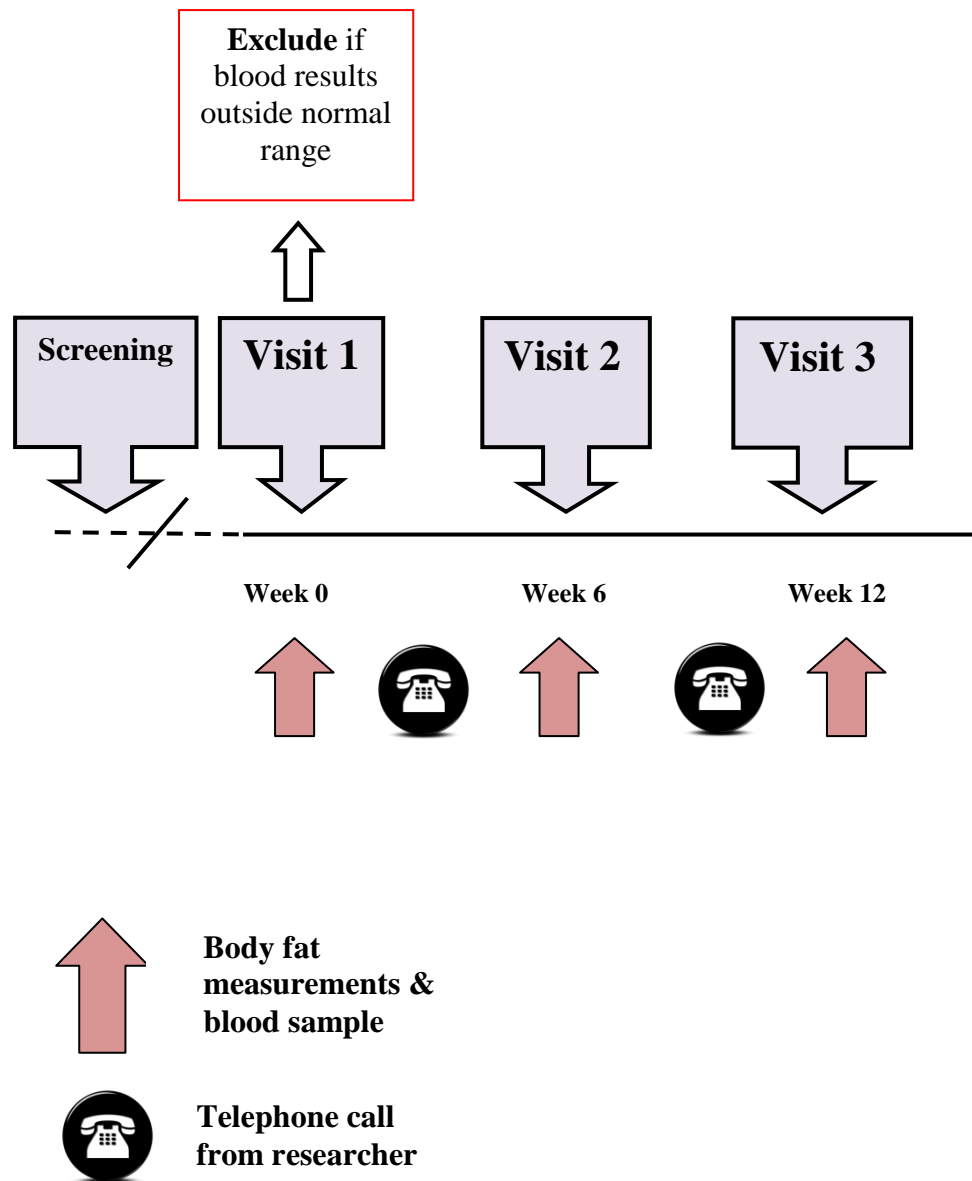
- I will ask you for information about your food intake in the previous 24 hours.

I will then organise collection/delivery of supplements to you. These will be either CLA or placebo in capsule form. You will be asked to take four 1g capsules per day with or after food (two capsules twice per day) for 12 weeks. These capsules are approximately the size of a kidney bean each. In order for the results to be valid, it is very important that you take the correct amount of capsules every day for the full 12 weeks. I will obtain details about your HIV diagnosis including years with HIV, HAART regimen, years since HAART started, changes in HAART since then, any other medications, or other diseases from the dietetic team, if possible, or from your medical notes.

I will contact you by telephone on weeks 2 and 4 to answer any questions you may have and to check your progress. On week 6 you will be asked to return to the Harrison Wing. During this visit, you will have your weight, waist circumference and body composition measured as in the first visit. I will ask you to complete a physical activity questionnaire and I will ask you for information about your food intake in the previous 24 hours. I will also ask you whether you have had any side-effects/symptoms as a result of the supplements. A 5 ml blood sample will be taken by a nurse or trained phlebotomist to measure the tests mentioned above. This visit should last 1 hour. I will also contact you at weeks 8 and 10 by telephone to answer any questions and to check your progress.

After 12 weeks, you will be asked to return for a third visit lasting approximately 1 hour. During this visit your weight, waist circumference and body fat composition will be measured as in previous visits and I will ask you to complete a physical activity questionnaire. I will ask you for information about your food intake in the previous 24 hours and ask you whether you have had any side-effects/symptoms as a result of the supplements. A 5 ml blood sample will be taken by a nurse or trained phlebotomist to measure all the tests as previously indicated. The overall study design is shown in the diagram below.

Study design



What are the possible side-effects of the supplements?

According to the European Food Safety Authority, the supplements are safe when given at a dose of 4 capsules per day for 12 weeks. However, there may be a small risk of diarrhoea, soft stools and stomach pain. In rare cases certain blood measures of liver function may increase. In rare cases, levels of free radicals in the body may increase; free radicals may cause damage to cells.

If you choose to take part in the study, we will monitor your progress closely and if you have any side-effects we will inform your doctor at Harrison Wing who will decide whether or not you should continue with the study.

Will your participation be kept confidential?

Information collected about you during this research will be kept strictly confidential and anonymity will be observed throughout the study by using a study ID in place of your name. Your name will be linked to this code in a separate file which will be kept locked in a secure place. Your name will only ever be linked to your study ID if you wish to withdraw from the study or we need to contact you or your GP to report blood results. Only the investigators have access to this data. Your GP will only be informed of your participation in the study if you would like us to inform them or if we find an abnormal blood result, in which case we will communicate this result to you, then to your GP and the team at Harrison Wing.

A summary of the group results of the study will be made available once the study is finished. Your personal details will not be identified in this report. Individual results will only be provided if you have specifically requested to receive your results at the start of the study.

What will happen to your study results?

We hope to publish the results of the study in a scientific journal. You will not be identified in any publication. We will be happy to discuss the overall results with you when the study is completed, and will let you know how you can get a copy of the published results if you wish. Future studies may investigate other effects of CLA using your sample, but in such cases, confidentiality and anonymity will be maintained and it will not be possible to identify you from any publication.

Who is organising and funding the study?

The study is organised by Cathriona Loonam and her PhD supervisor, Dr Anne Mullen, at the Diabetes and Nutritional Sciences Division, Kings College London. Neither Miss Loonam nor Dr Mullen will be paid for recruiting participants to the study. The study is funded by a King's College London PhD scholarship provided as part of the researcher's PhD programme. The supplements will be provided by Stepan Lipid Nutrition, a nutrition company based in the Netherlands. In recognition of your time commitment, you will be paid an honorarium of £50 upon completion of the study and you will be refunded the cost of travelling to and from the Harrison Wing for each visit.

Do you have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You can withdraw from the study at any time by informing one of the researchers and you are not obliged to give a reason. You can withdraw your data from the study up until the time of publication (December 2014). A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions [020 7848 4081]. If you remain unhappy and wish to complain formally, you can do this by contacting either NHS England (england.contactus@nhs.net) or your local Clinical Commissioning Group (<http://www.nhs.uk/Service-Search/Clinical-Commissioning-Group/LocationSearch/1>)

Further information and contact details

If you would like further information or would like to take part, please contact the researcher, Cathriona Loonam, by email (claartstudy@kcl.ac.uk) or by telephone (020 7848 4162), or her supervisor Dr Anne Mullen by email (anne.mullen@kcl.ac.uk) or by telephone (020 7848 4081).

If you decide to take part please let me know if you have been involved in any other study in the last year.

Thank you for your interest.

Appendix 7 CLAART Study consent form



CONSENT FORM FOR PARTICIPANTS IN A NUTRITIONAL STUDY

Please complete this form after you have read the Information Sheet and you are satisfied that the research has been fully explained.

Title of Study: A pilot study to investigate the effects of a novel dairy fat (conjugated linoleic acid; CLA) on levels of body fat in men with HIV receiving HAART: the CLAART study

Research Ethics Committee Ref: 13/LO/1145

Thank you for considering taking part in this research. The person organising the research must explain the project to you before you agree to take part. If you have any questions arising from the Information Sheet or explanation already given to you, please ask the researcher before you decide whether to participate. You will be given a copy of this Consent Form to keep and refer to at any time.

I confirm that I fit into the following criteria:

I am a male aged between 18 and 55 years ☐

I do not have a history of heart disease, stroke, blood clots, diabetes, liver or kidney diseases, intestinal problems, hepatitis C or a cancer diagnosis (except basal cell carcinoma) ☐

I do not consume excess alcohol intake (excess = >21 units per week) or use illicit drugs ☐

I think I may have a large waist circumference or a high body mass index (BMI) ☐

I understand that if I decide at any time during the research that I no longer wish to participate in this project, I can notify the researchers involved and be withdrawn from it immediately. Furthermore, I understand that I will be able to withdraw my data up to the point of publication.

I consent to the processing of my personal information for the purposes of this research study. I understand that such information will be treated as strictly confidential and handled in accordance with the provisions of the Data Protection Act 1998.

I agree that the research team of Cathriona Loonam and Dr Anne Mullen may use my samples for future research. Please note that in such cases, as with this project, confidentiality and anonymity will be maintained and it will not be possible to identify you from any publication.

Participant's Statement: I _____
agree that the research project named above has been explained to me to my satisfaction and I agree to take part in the study. I have read both the notes written above and the Information Sheet about the project, and understand what the research study involves.

Signed _____ Date _____

Investigator's Statement: I _____
confirm that I have carefully explained the nature, demands and any foreseeable risks (where applicable) of the proposed research to the volunteer.

Signed _____ Date _____

Appendix 8 CLAART study data collection sheet

CLAART study data collection sheet

Anthropometric assessment- Visit 01

Measurements

Height _____(cm)

Weight _____(kg)

Reported usual weight _____(kg) / _____(stones, pounds)

Reported weight loss in the previous 6 months _____(kg)

Body mass index (BMI) _____(kg/m²)

Waist circumference _____(cm)

Upper arm length _____(cm)

Upper arm circumference _____(cm)

Tricep skin fold thickness (1) _____ (2) _____ (3) _____
(Avg.) _____(mm)

Bicep skin fold thickness (1) _____ (2) _____ (3) _____
(Avg.) _____(mm)

Supra-iliac skinfold (1) _____ (2) _____ (3) _____
(Avg.) _____(mm)

Subscapular skinfold (1) _____ (2) _____ (3) _____
(Avg.) _____(mm)

Total skinfolds: _____ **log Total:** _____

Body Density ($D \times 10^3 \text{kg/m}^3$): Adult males (16-19y) = $1.1620 - 0.0630 \times \log_{10}x$

Adult males (20-29y) = $1.1631 - 0.0632 \times \log_{10}x$

Adult males (30-39y) = $1.1422 - 0.0544 \times \log_{10}x$

Adult males (40-49y) = $1.1620 - 0.0700 \times \log_{10}x$

Adult males (50+) = $1.1715 - 0.0779 \times \log_{10}x$

Fat (%) = $4.95 / D - 4.50 \times 100$ _____

Appendix 9 Letter to general practitioner



[Doctor Name]

[Medical Practice or Hospital Name]

[Address]

RE: [Patient Name] participation in clinical trial

Date of birth: [DOB], NHS number: [NHS number]

Dear [Doctor Name]:

I am writing to inform you that [Patient Name] has requested that you should be informed of his participation in our research trial: A pilot study to investigate the effects of a novel dairy fat (conjugated linoleic acid; CLA) on levels of body fat in men with HIV receiving HAART: The CLAART Study. This trial is being conducted at the Harrison Wing, St Thomas' Hospital, as part of a PhD project funded by King's College London.

This is a pilot, randomised, double-blind, placebo-controlled trial investigating the effects of CLA supplementation on measures of body fat including waist circumference. Participants are required to take four 1g CLA capsules (3g CLA total) per day for 12 weeks. The primary outcome of the study is a reduction in waist circumference. Participants will attend three visits at the Harrison Wing where they will have their height, weight, waist circumference and body fat distribution measured, as well as blood samples taken to measure triglycerides, total cholesterol, glucose, liver function, oxidative stress, CD4 count and viral load, as measures of the potential side effects of CLA. As a measure of adherence, plasma CLA levels will also be measured. Any clinically significant findings during this trial will be communicated to you, but if you have any questions, please do not hesitate to contact me by telephone 0207 848 4162 or email claartstudy@kcl.ac.uk.

Sincerely,

Cathriona Loonam

Diabetes & Nutritional Sciences Division
King's College London
Franklin Wilkins Building
150 Stamford Street
London
SE1 9NH

Appendix 10 Body Density Equations

Adult males body density ($D \times 10^3 \text{ kg/m}^3$):

$$(16-19y) = 1.1620 - 0.0630 \times \log_{10}x$$

$$(20-29y) = 1.1631 - 0.0632 \times \log_{10}x$$

$$(30-39y) = 1.1422 - 0.0544 \times \log_{10}x$$

$$(40-49y) = 1.1620 - 0.0700 \times \log_{10}x$$

$$(50+) = 1.1715 - 0.0779 \times \log_{10}x$$

(Durnin and Womersley 1974)

Appendix 11 EPIC Norfolk Food Frequency Questionnaire

FOOD FREQUENCY QUESTIONNAIRE

This questionnaire asks for some background information about you, especially about what you eat.

Please answer every question. If you are uncertain about how to answer a question then do the best you can, but please do not leave a question blank.

1. **YOUR DIET LAST YEAR**

For each food there is an amount shown, either a "medium serving" or a common household unit such as a slice or teaspoon. Please put a tick (✓) in the box to indicate how often, **on average**, you have eaten the specified amount of each food **during the past year**.

EXAMPLES:

For white bread the amount is one slice, so if you ate 4 or 5 slices a day, you should put a tick in the column headed "4-5 per day".

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
BREAD AND SAVOURY BISCUITS (one slice or biscuit)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
White bread and rolls								✓	

For chips, the amount is a "medium serving", so if you had a helping of chips twice a week you should put a tick in the column headed "2-4 per week".

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
POTATOES, RICE AND PASTA (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Chips				✓					

For very seasonal fruits such as strawberries and raspberries you should estimate your average use when the fruits are in season, so if you ate strawberries or raspberries about once a week when they were in season you should put a tick in the column headed "once a week".

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
FRUIT (1 fruit or medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Strawberries, raspberries, kiwi fruit			✓						

Please estimate your average food use as best you can, and please answer every question - do not leave ANY lines blank. PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
MEAT AND FISH (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Beef: roast, steak, mince, stew or casserole									
Beefburgers									
Pork: roast, chops, stew or slices									
Lamb: roast, chops or stew									
Chicken or other poultry eg. turkey									
Bacon									
Ham									
Corned beef, Spam, luncheon meats									
Sausages									
Savoury pies, eg. meat pie, pork pie, pasties, steak & kidney pie, sausage rolls									
Liver, liver pâté, liver sausage									
Fried fish in batter, as in fish and chips									
Fish fingers, fish cakes									
Other white fish, fresh or frozen, eg. cod, haddock, plaice, sole, halibut									
Oily fish, fresh or canned, eg. mackerel, kippers, tuna, salmon, sardines, herring									
Shellfish, eg. crab, prawns, mussels									
Fish roe, taramasalata									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
BREAD AND SAVOURY BISCUITS (one slice or biscuit)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
White bread and rolls									
Brown bread and rolls									
Wholemeal bread and rolls									
Cream crackers, cheese biscuits									
Crispbread, eg. Ryvita									
CEREALS (one bowl)									
Porridge, Readybrek									
Breakfast cereal such as cornflakes, muesli etc.									
POTATOES, RICE AND PASTA (medium serving)									
Boiled, mashed, instant or jacket potatoes									
Chips									
Roast potatoes									
Potato salad									
White rice									
Brown rice									
White or green pasta, eg. spaghetti, macaroni, noodles									
Wholemeal pasta									
Lasagne, moussaka									
Pizza									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
DAIRY PRODUCTS AND FATS	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Single or sour cream (tablespoon)									
Double or clotted cream (tablespoon)									
Low fat yogurt, fromage frais (125g carton)									
Full fat or Greek yogurt (125g carton)									
Dairy desserts (125g carton)									
Cheese, eg. Cheddar, Brie, Edam (medium serving)									
Cottage cheese, low fat soft cheese (medium serving)									
Eggs as boiled, fried, scrambled, etc. (one)									
Quiche (medium serving)									
Low calorie, low fat salad cream (tablespoon)									
Salad cream, mayonnaise (tablespoon)									
French dressing (tablespoon)									
Other salad dressing (tablespoon)									
The following on bread or vegetables									
Butter (teaspoon)									
Block margarine, eg. Stork, Krona (teaspoon)									
Polyunsaturated margarine (tub), eg. Flora, sunflower (teaspoon)									
Other soft margarine, dairy spreads (tub), eg. Blue Band, Clover (teaspoon)									
Low fat spread (tub), eg. Outline, Gold (teaspoon)									
Very low fat spread (tub) (teaspoon)									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
SWEETS AND SNACKS (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Sweet biscuits, chocolate , eg. digestive (one)									
Sweet biscuits, plain, eg. Nice, ginger (one)									
Cakes eg. fruit, sponge, home baked									
Cakes eg. fruit, sponge, ready made									
Buns, pastries eg. scones, flapjacks, home baked									
Buns, pastries eg. croissants, doughnuts, ready made									
Fruit pies, tarts, crumbles, home baked									
Fruit pies, tarts, crumbles, ready made									
Sponge puddings, home baked									
Sponge puddings, ready made									
Milk puddings, eg. rice, custard, trifle									
Ice cream, choc ices									
Chocolates, single or squares									
Chocolate snack bars eg. Mars, Crunchie									
Sweets, toffees, mints									
Sugar added to tea, coffee, cereal (teaspoon)									
Crisps or other packet snacks, eg. Wotsits									
Peanuts or other nuts									
SOUPS, SAUCES, AND SPREADS									
Vegetable soups (bowl)									
Meat soups (bowl)									
Sauces, eg. white sauce, cheese sauce, gravy (tablespoon)									
Tomato ketchup (tablespoon)									
Pickles, chutney (tablespoon)									
Marmite, Bovril (teaspoon)									
Jam, marmalade, honey (teaspoon)									
Peanut butter (teaspoon)									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
DRINKS	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Tea (cup)									
Coffee, instant or ground (cup)									
Coffee, decaffeinated (cup)									
Coffee whitener, eg. Coffee-mate (teaspoon)									
Cocoa, hot chocolate (cup)									
Horlicks, Ovaltine (cup)									
Wine (glass)									
Beer, lager or cider (half pint)									
Port, sherry, vermouth, liqueurs (glass)									
Spirits, eg. gin, brandy, whisky, vodka (single)									
Low calorie or diet fizzy soft drinks (glass)									
Fizzy soft drinks, eg. Coca cola, lemonade (glass)									
Pure fruit juice (100%) eg. orange, apple juice (glass)									
Fruit squash or cordial (glass)									
FRUIT									
For seasonal fruits marked *, please estimate your average use when the fruit is in season									
Apples (1 fruit)									
Pears (1 fruit)									
Oranges, satsumas, mandarins (1 fruit)									
Grapefruit (half)									
Bananas (1 fruit)									
Grapes (medium serving)									
Melon (1 slice)									
* Peaches, plums, apricots (1 fruit)									
* Strawberries, raspberries, kiwi fruit (medium serving)									
Tinned fruit (medium serving)									
Dried fruit, eg. raisins, prunes (medium serving)									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

PLEASE PUT A TICK (✓) ON EVERY LINE

FOODS AND AMOUNTS	AVERAGE USE LAST YEAR								
VEGETABLES Fresh, frozen or tinned (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Carrots									
Spinach									
Broccoli, spring greens, kale									
Brussels sprouts									
Cabbage									
Peas									
Green beans, broad beans, runner beans									
Marrow, courgettes									
Cauliflower									
Parsnips, turnips, swedes									
Leeks									
Onions									
Garlic									
Mushrooms									
Sweet peppers									
Beansprouts									
Green salad, lettuce, cucumber, celery									
Watercress									
Tomatoes									
Sweetcorn									
Beetroot									
Coleslaw									
Avocado									
Baked beans									
Dried lentils, beans, peas									
Tofu , soya meat, TVP, Vegeburger									
	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick (✓) on EVERY line

YOUR DIET LAST YEAR, continued

2. Are there any **OTHER** foods which you ate more than once a week? Yes ☐ No ☐

If yes, please list below

Food	Usual serving size	Number of times eaten each week
<input type="text"/>	<input type="text"/>	<input type="text"/>
<input type="text"/>	<input type="text"/>	<input type="text"/>
<input type="text"/>	<input type="text"/>	<input type="text"/>
<input type="text"/>	<input type="text"/>	<input type="text"/>
<input type="text"/>	<input type="text"/>	<input type="text"/>
<input type="text"/>	<input type="text"/>	<input type="text"/>

3. What type of milk did you most often use?

Select one only

Full cream, silver ☐

Semi-skimmed, red/white ☐

Skimmed/blue ☐

Channel Islands, gold ☐

Dried milk ☐

Soya ☐

Other, specify

None ☐

4. How much milk did you drink each day, including milk with tea, coffee, cereals etc?

None ☐

Three quarters of a pint ☐

Quarter of a pint ☐

One pint ☐

Half a pint ☐

More than one pint ☐

5. Did you usually eat breakfast cereal (excluding porridge and Ready Brek mentioned earlier)?

Yes ☐ No ☐

If yes, which brand and type of breakfast cereal, including muesli, did you usually eat?

List the one or two types most often used

Brand e.g. Kellogg's

Type e.g. cornflakes

6. What kind of fat did you most often use for frying, roasting, grilling etc?

Select one only

Butter ☐

Solid vegetable fat ☐

Lard/dripping ☐

Margarine ☐

Vegetable oil ☐

None ☐

If you used vegetable oil, please give type eg. corn, sunflower

7. What kind of fat did you most often use for baking cakes etc?

Select one only

Butter ☐

Solid vegetable fat ☐

Lard/dripping ☐

Margarine ☐

Vegetable oil ☐

None ☐

If you used margarine, please give name or type eg. Flora, Stork

8. How often did you eat food that was fried at home?
 Daily ☐ 1-3 times a week ☐ 4-6 times a week ☐
 Less than once a week ☐ Never ☐
9. How often did you eat fried food away from home?
 Daily ☐ 1-3 times a week ☐ 4-6 times a week ☐
 Less than once a week ☐ Never ☐
10. What did you do with the visible fat on your meat?
 Ate most of the fat ☐ Ate as little as possible ☐
 Ate some of the fat ☐ Did not eat meat ☐
11. How often did you eat grilled or roast meat? times a week
12. How well cooked did you usually have grilled or roast meat?
 Well done /dark brown ☐ Lightly cooked/rare ☐
 Medium ☐ Did not eat meat ☐
13. How often did you add salt to food while cooking?
 Always ☐ Rarely ☐
 Usually ☐ Never ☐
 Sometimes ☐
14. How often did you add salt to any food at the table?
 Always ☐ Rarely ☐
 Usually ☐ Never ☐
 Sometimes ☐
15. Did you regularly use a salt substitute (eg LoSalt)? Yes ☐ No ☐
 If yes, which brand?
16. During the course of last year, on average, how many times a week did you eat the following foods?
- | Food type | Times/week | Portion size |
|--|----------------------|---------------------------|
| Vegetables (not including potatoes) | <input type="text"/> | medium serving |
| Salads | <input type="text"/> | medium serving |
| Fruit and fruit products (not including fruit juice) | <input type="text"/> | medium serving or 1 fruit |
| Fish and fish products | <input type="text"/> | medium serving |
| Meat, meat products and meat dishes (including bacon, ham and chicken) | <input type="text"/> | medium serving |

17. Have you taken any vitamins, minerals, fish oils, fibre or other food supplements during the past year? Yes ☐ No ☐ Don't know ☐

If **yes**, please complete the table below. If you have taken more than 5 types of supplement please put the most frequently consumed brands first.

Vitamin supplements		Average frequency								
		Tick one box per line to show how often on average you consumed supplements								
Name and brand Please list full name, brand and strength	Dose Please state number of pills, capsules or teaspoons consumed	Never or less than once a month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Thank you for your help

Appendix 12 24 hour recall instructions

Acknowledgments must be given to Nelson M, Erens B, Bates B, Church S & Boshier T if this questionnaire is used or modified.

24-HOUR RECALL INSTRUCTIONS

OBTAINING A 24-HOUR RECALL

The 24-hour recall aims to provide a complete record of all food and drink eaten on the previous day between midnight and midnight. The time element is important, as there may be respondents who work shifts or that have unusual time schedules meaning that their dietary patterns are not typical.

The 24-hour recall is collected in three phases (Triple Pass):

1. A quick list of foods eaten or drunk.

Respondents are asked to report everything that they had to eat or drink on the previous day between midnight and midnight. This recall session is not interrupted. At the end of the recall, respondents are invited to add any additional items not initially recalled.

2. Collection of detailed information concerning the items in the quick list.

For each item of food or drink, respondents are asked to provide additional detail.

- a) The time at which the food or drink was consumed.
- b) A full description of the food or drink, including brand name where available.
- c) Any foods likely to be eaten in combination e.g. milk in coffee
- d) Recipes and other combinations of foods e.g. sandwiches
- e) The quantity consumed, based on household measures, photographs of different portion sizes of foods or weights.
- f) Any leftovers or second helpings.

3. A recall review in which respondents are given an opportunity to provide additional information and for the interviewer to prompt for information about foods or drink not mentioned.

The interviewer reviews all the food eaten and drunk in chronological order, prompts for any additional eating or drinking occasions or foods/drink possibly consumed and clarifies any ambiguities regarding type of food eaten or portion size. Finally the interviewer asks the respondent to name the place where each food or drink item was consumed. All of the information gathered is recorded by the interviewer on the record sheet in the booklet called Food Consumption Record: Individual 24-hour recall.

**Protocol for the completion of a food consumption record:
Individual 24-hour recall**

The following instructions provide detailed prompts for obtaining information in the individual 24-hour recall of food and drink consumption. Please follow the sequence carefully. Where words appear in upper case inside parentheses, supply the appropriate word (e.g. if DAY appears, say the name of the appropriate day of the week). Words that appear inside square brackets are instructions to you.

Introduction at beginning of first 24 hour recall

[Where interview being conducted with parent/carer on behalf of child, insert child's name as appropriate]

IF RESPONDENT IS ADULT OR CHILD AGED UNDER FIVE SAY

I am going to ask you about everything that you/(NAME OF CHILD) ate and drank yesterday. By this I mean, 24 hours from midnight to midnight. I would like to know exactly what was eaten and drunk and how much you/(NAME OF CHILD) had.

OR

IF RESPONDENT IS CHILD AGED BETWEEN FIVE AND TWELVE SAY

I am going to ask you about everything that (NAME OF CHILD) ate and drank yesterday. By this I mean, 24 hours from midnight to midnight. I would like to know exactly what was eaten and drunk and how much (NAME OF CHILD) had.

[Addressing yourself to child, say]

I will ask you to tell me about any foods and drinks which your (MUM, DAD (main food provider)) does not know about or cannot remember. Is that okay?

[Wait for agreement].

OR

IF RESPONDENT IS CHILD AGED TWELVE AND OVER SAY

I am going to ask you about everything that you ate and drank yesterday. By this I mean, 24 hours from midnight to midnight. I would like to know exactly what was eaten and drunk and how much you had.

[Say to Mum or Dad]

I would prefer to ask (NAME OF CHILD) for information first and then ask you to fill in the details. Is that okay? [Wait for agreement].

[Addressing yourself to child say]

I will ask your (MUM, DAD (main food provider)) to tell me exactly what it was you ate when you are not sure.

Introducing portion size estimation

[Where interview being conducted with parent/carer on behalf of child, insert child's name as appropriate]

THE INTERVIEWER SAYS:

1. When I ask you how much food and drink you/(NAME OF CHILD) had, I would like you to tell me in as much detail as possible in terms of the size of the package for example half a tin of baked beans. In this case also tell me the size of the tin for example a 420g tin.
2. Or in terms of household utensils for example a glass of milk. In this case I would like you to tell me how big the glass was. Or number of spoons, in which case I would ask you for the size of spoon. This is a life size photograph of a teaspoon, dessertspoon and tablespoon [show photograph of spoons]
3. To help you to tell me how much of a food you/(NAME OF CHILD) ate, I have a book here with photographs of different amounts of foods.
[Show book and open book at page 1 (photograph of rice)]
As you can see there are eight photographs. I will ask you to pick **one photograph** that looks like the amount you/(NAME OF CHILD) had to eat.
4. This is a life size photograph of the plate used in most of these photographs.
[Show photograph of 10" plate]
[Show other photographs of plates, as necessary during recall]
5. Have a look at a few more photographs and then we can start.
[Allow subject to flick through book if they want and start when they are ready].
6. If you/(NAME OF CHILD) ate any homemade dishes for example a stew, I would like you to tell me the ingredients and how much was used. If you do not know the ingredients I may need to ask whoever prepared and cooked the dish

24 hour recall itself

THE INTERVIEWER SAYS:

1. I would like you to tell me everything that you had to eat and drink yesterday. By yesterday I mean, from midnight to midnight. Include everything that you/(NAME OF CHILD) had to eat and drink at home and away from home, including snacks, tea, coffee, sweets, soft drinks (AND ALCOHOL). [Omit for child].
 - First we'll make a list of the foods you/(NAME OF CHILD) ate and drank all day yesterday (DAY).
 - Next I'll ask you about the foods including amounts and then I'll ask you a few questions.
 - It may help you to remember what you/(NAME OF CHILD) ate by thinking about where you/(NAME OF CHILD) were, whom you/(NAME OF CHILD) were with, or what you/(NAME OF CHILD) were doing yesterday; like going to work, eating out or watching television. Feel free to keep these activities in mind and say them aloud if it helps you.
 - So... if you would like to start at midnight at the beginning of (DAY).

[COMPLETE QUICK LIST WITHOUT INTERRUPTION]

[WHEN SUBJECT STOPS ASK]

- What else?

2. [Where interview is being addressed to child]
[Say to parent/carer] Can you think of anything else that (NAME OF CHILD) had to eat or drink yesterday.

OR

2. [Where interview is being addressed to parent on behalf of child]
[Say to child] Can you think of anything else that you had to eat or drink yesterday?

[ADD ITEMS INTO QUICK LIST AT APPROPRIATE POINTS]

[THEN ASK]

- What else?

[CONTINUE UNTIL NO FURTHER ADDITIONS]

3. There are some foods that people often forget. In addition to what you have already told me about, did you/(NAME OF CHILD) have any:
 - Coffee, tea, soft drinks or milk
 - Alcoholic drinks
 - Biscuits, cakes, sweets, chocolate bars or other confectionery
 - Crisps, peanuts or other snacks
 - Sauces, dressings,
 - Anything you have not already told me about?

4. Now I would like to go through the list you have just given me and ask you some details about each item of food and drink. If while we are talking you remember anything else that you/(NAME OF CHILD) had to eat or drink, please tell me.

4a. Was (FIRST FOOD FROM QUICK LIST) the first thing that you/(NAME OF CHILD) had to eat/drink yesterday?

IF YES: [GO TO BOX 1, Step a and work through steps in box]

IF NO: What was the first thing you/(NAME OF CHILD) had to eat or drink yesterday?

[RECORD ITEM NAMED ON MAIN LIST]

[GO TO BOX 1, Step b]

4b. Was (NEXT ITEM FROM QUICK LIST) the next thing you/(NAME OF CHILD) had to eat/drink?

[CONFIRM IF FOOD IS OBVIOUSLY PART OF SAME MEAL (e.g. milk on cereal) AND GO TO BOX 1]

4c. **[CONTINUE UNTIL ALL FOODS ON QUICK LIST HAVE BEEN TICKED]**

BOX 1

- a. **TRANSFER ITEM FROM QUICK LIST AND TICK BOX.**
- b. (If necessary) **ASK:** About what time was that?
- c. **RECORD TIME** (in 24-hour clock format e.g. 18.00 for 6pm)
- d. **ASK FOR DETAILED DESCRIPTION (USE THE FOOD DESCRIPTIONS LISTED AT THE BEGINNING OF THE RECORD SHEET AND THE FOOD DESCRIPTION PROMPT SHEET)**
- e. **RECORD 'DESCRIPTION'**
- f. **ASK FOR BRAND NAME**
- g. **RECORD 'BRAND NAME' (if recalled at first request)**
- h. **ASK FOR AMOUNT (USE PHOTOS, HOUSEHOLD MEASURES OR WEIGHTS)**
- i. **RECORD 'AMOUNT'**
- j. (If necessary) **PROMPT FOR RECIPES. (Record on recipe pages including amounts of ingredients)**
- k. **GO TO 4b**

Before moving on to the next meal/snack:

- l. **ASK ABOUT SECOND HELPINGS. (Record on separate line)**
- m. **ASK ABOUT LEFTOVERS (Record in Leftovers column).**
- n. (If necessary) **PROMPT FOR ADDITIONS (USE COMMONLY CONSUMED ADDITIONAL FOOD PROMPTS)**

REVIEW

5. Let's see if I have everything. I would like you to try and remember anything else that you/(NAME OF CHILD) had to eat or drink yesterday that you have not already told me about, including anything that you/(NAME OF CHILD) had to eat or drink while you were preparing a meal or waiting to eat.

[USE THE FOLLOWING PROMPTS TO ELICIT ADDITIONAL FOODS]

- 5a. Did you/(NAME OF CHILD) have anything to eat or drink between midnight yesterday and (TIME / NAME OF FIRST OCCASION)?
- 5b. At (TIME / NAME OF OCCASION) you/(NAME OF CHILD) had (FOODS/DRINKS). Do you recall (NAME OF CHILD) having anything else to eat or drink?
- 5c. Did you/(NAME OF CHILD) have anything to eat or drink between (TIME / THIS OCCASION) and (NEXT OCCASION)?

[REPEAT STEPS 5b TO 5c UNTIL LAST OCCASION / TIME]

- 5d. At (TIME / NAME OF OCCASION) you/(NAME OF CHILD) had (FOODS/DRINKS). Do you recall (NAME OF CHILD) having anything else to eat or drink?
- 5e. Did you/(NAME OF CHILD) have anything else to eat or drink between (THIS OCCASION) and midnight last night?

PLACE NAMES

6. I would like to ask you to give me a place name from this card **[SHOW CARD 1]** for each occasion at which you/(NAME OF CHILD) ate or drank something.

[FOR EACH OCCASION / TIME ASK:]

- 6a. Where did you/(NAME OF CHILD) eat/drink that?

[PROMPT WITH CARD 1]

- 6b. **[ENTER "PLACE" LETTER]**

- 7 **[WHERE BRAND HAS NOT BEEN RECALLED AT FIRST REQUEST BUT RESPONDENT HAS PRODUCT IN CUPBOARD, FRIDGE ETC, ASK IF YOU CAN CHECK PRODUCT AND ENTER BRAND NAME ON RECALL]**

- 8 **[COMPLETE '24 HOUR QUESTIONS' IN THE BOOKLET FOR THIS FOOD CONSUMPTION RECORD]**

Appendix 13 International Physical Activity Questionnaire (Short Version)

PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activity you do as part of your everyday life because it may affect your waist circumference, weight or body fat levels. The questions are about the time you spent being physically active in the last 7 days. They include questions about activities you do at work, as part of your house and garden work, to get from place to place, and in your spare time for recreation, exercise or sport.

Your answers are important.

Please answer each question even if you do not consider yourself to be an active person.

In answering the following questions,

➤ **vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal.

➤ **moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

1a) During the last 7 days, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling?

Think about *only* those physical activities that you did for at least 10 minutes at a time.

_____ **days per week** ➡

1b. How much time in total did you usually spend on one of those days doing vigorous physical activities?

_____ **hours** _____ **minutes**

or none ☐

2a. Again, think *only* about those physical activities that you did for at least 10 minutes at a time.

During the last 7 days, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

PHYSICAL ACTIVITY QUESTIONNAIRE *continued*

_____ **days per week** \Rightarrow 2b. How much time in total did you usually
spend on one of those days doing moderate
physical activities?
_____ **hours** _____ **minutes**
or none ☐

3a. During the last 7 days, on how many days did you **walk** for at least 10 minutes at a time? This includes walking at work and at home, walking to travel from place to place, and any other walking that you did solely for recreation, sport, exercise or leisure.

_____ **days per week** \Rightarrow 3b. How much time in total did you usually
spend walking on one of those days?
_____ **hours** _____ **minutes**
or none ☐

The last question is about the time you spent sitting on weekdays while at work, at home, while doing course work and during leisure time. This includes time spent sitting at a desk, visiting friends, reading travelling on a bus or sitting or lying down to watch television.

4. During the last 7 days, how much time in total did you usually spend *sitting* on a **weekday**? _____ **hours** _____ **minutes**

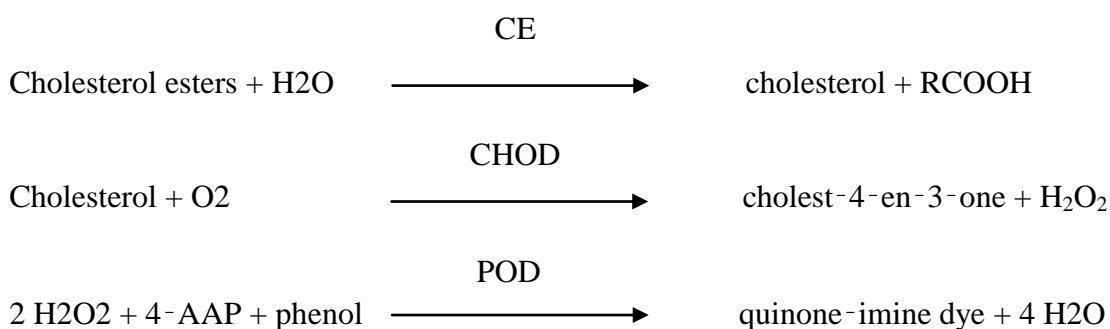
Appendix 14 Determination of serum total cholesterol

Sample requirements

The Roche c8000 cobas c system used 2 µl of sample in the test.

Method

Serum cholesterol concentration was determined on the Roche cobas c8000 system (Roche Diagnostics Ltd., Burgess Hill, West Sussex, UK). Cholesterol esters were cleaved by the action of cholesterol esterase (CE) to yield free cholesterol and fatty acids. Free cholesterol was oxidised to cholesten-3-one and hydrogen peroxide by cholesterol oxidase (CHOD). Peroxidase (POD) catalysed the reaction of hydrogen peroxide with 4-aminoantipyrine and phenol to produce a red coloured quinoneimine product. Colour intensity is directly proportional to concentration of cholesterol and was measured spectrophotometrically at 520 nm. Intra-assay CV was 0.8 % and inter-assay CV was 1.5 %.



Appendix 15 Determination of serum HDL-cholesterol

Sample requirements

The Roche c8000 cobas system used 2 µl of sample in the test.

Method

HDL-C concentration in serum was determined using Roche colorimetric assay on the cobas c8000 system. The Roche assay for the measurement of HDL-C is an enzymatic assay. The cholesterol concentration of HDL-C was determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with PEG to the amino group. Cholesterol esters are broken down quantitatively into free cholesterol and fatty acids by cholesterol esterase. In the presence of oxygen, cholesterol was oxidised by cholesterol oxidase to Δ^4 -cholestenone and hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide generated reacted with 4-amino-antipyrine and HSDA to form a purple-blue dye. The colour intensity of this dye is directly proportional to the cholesterol concentration and was measured spectrophotometrically at 600 nm. Intra-assay CV was 0.7 % and inter-assay CV was 0.9 %.

Appendix 16 Determination of triglyceride levels

Sample requirements

The Roche c8000 cobas system used 2 µl of sample in the test.

Method

Triglyceride concentration in plasma samples was determined using the Roche c8000 system. The Roche assay for the measurement of TG is an enzymatic colorimetric assay. Triglycerides in the sample were hydrolyzed to glycerol and free fatty acids by the action of lipase. A sequence of three coupled enzymatic steps using glycerol kinase, glycerophosphate oxidase, and horseradish peroxidase causes oxidative coupling of 3,5-dichloro-2-hydroxybenzenesulfonic acid with 4-aminoantipyrine to form a red quinoneimine dye. The change in absorbance (520 nm) is directly proportional to the concentration of triglycerides in the sample. Intra-assay CV was 0.7 % and inter-assay CV was 1.8 %.

Appendix 17 Determination of fasting glucose levels

Sample requirements

Samples were taken into fluoride-oxalate tubes. The Roche cobas c8000 system used 2 µl of sample in the test.

Methods

Glucose concentration in fasting plasma samples was determined using a Roche colorimetric assay on a cobas c8000 system. Glucose was oxidised by glucose oxidase to gluconolactone in the presence of atmospheric oxygen. The resultant hydrogen peroxide oxidised 4-aminophenazone and phenol to 4-benzoquinone-monoimino phenazone in the presence of peroxidase. The colour intensity of the red dye is directly proportional to the glucose concentration and was measured spectrophotometrically at 340 nm. Intra-assay CV was 0.5 % and the inter-assay CV was 1.2%.

Appendix 18 Determination of liver function

Sample requirements

The sample was taken into a serum separator tube. The Roche cobas c8000 system used 2 µl of sample in each test.

Methods

Albumin

At a pH of 4.1, bromcresol green (BCG), an anionic dye, binds selectively with cationic albumin to form an albumin-BCG complex. The colour intensity of the blue-green colour was directly proportional to the albumin concentration and determined spectrophotometrically at 570 nm. Intra-assay CV was 0.5 % and inter-assay CV was 0.95 %.

ALT

ALT catalyzes the reversible transamination of L-alanine and 2-oxoglutarate to pyruvate and L-glutamate. Pyruvate is then reduced to lactate in the presence of lactate dehydrogenase with the concurrent oxidation of NADH to NAD. The rate of the NAD oxidation is directly proportional to the catalytic ALT activity. It was determined by measuring the decrease in absorbance at 340 nm. Intra-assay CV was 0.5 % and inter-assay CV was 2.95 %.

ALP

P-nitrophenyl phosphate is cleaved by phosphatases into phosphate and p-nitrophenol in the presence of magnesium and zinc ions. The p-nitrophenol released is directly proportional to the catalytic ALP activity and was determined by measuring the increase in absorbance at 480 nm. Intra-assay CV was 0.6 % and inter-assay CV was 1.6 %.

Bilirubin

Total bilirubin in the presence of caffeine, benzoate, and acetate is coupled with a diazonium ion in a strongly acidic medium. The intensity of the colour of the azobilirubin produced is proportional to the total bilirubin concentration and was measured spectrophotometrically at 520 nm. Intra-assay CV was 1.2 % and inter-assay CV was 1.6 %.

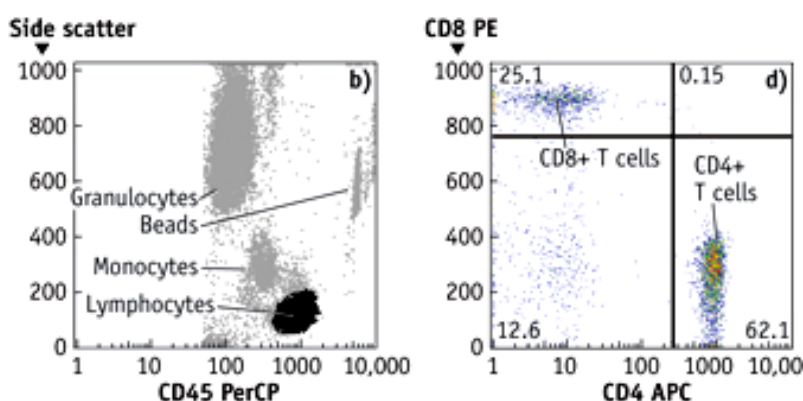
Appendix 19 Determination of CD4+ T lymphocyte count

Sample requirements

The sample was taken into an EDTA tube. The Beckman Coulter FP1000 system used 100 µl of sample in each test.

Method

Sample preparation was carried out in a closed system using the automated FP1000 cell preparation system (Beckman Coulter). 100 µl of the sample was injected into the FP1000 system, to which 10 µl of Cyto-Stat[®] containing a four-colour monoclonal antibody panel was added. The addition of CD45 to the tube containing CD4, CD8 and CD3 allowed for the identification of lymphocytes based on CD45 and side scatter and for enumeration of CD4 and CD8 cells. Using a single platform approach, 100 µl of Flow-Count[™] Fluorospheres were added to the CD4 stained sample. The fluorospheres contain a dye which emits fluorescence when excited at 488 nm. An assayed concentration of the fluorospheres allowed for the determination of absolute lymphocyte counts. The sample was incubated, fixed, lysed and stabilised on the FP1000 system. A no-wash method was used for single platform analysis. Flow-cytometric analysis was performed using a 5-colour flow cytometer equipped with FC500 CXP software (Cytomics FC 500, Beckman Coulter). A threshold was set according to manufacturer's recommendations, side-scatter was adjusted to identify leukocyte populations and a gate was set manually around the CD45 population to allow for analysis within this population. Lymphocyte populations were identified based on CD45 fluorescence and low side-scattering (see below).



Appendix 10 Determination of HIV viral load

Sample requirements

The sample was taken into an EDTA tube. The Roche COBAS® AmpliPrep/COBAS® TaqMan® HIV-1 Test v2.0 used 850 µl of sample for the test.

Methods

The AmpliPrep/COBAS® TaqMan® HIV-1 Test v2.0 was performed using the COBAS® TaqMan® Analyser. The quantitation of HIV-1 viral RNA was performed using HIV-1 Quantitation Standard (QS) Armored RNA. The HIV sequences in the QS contain identical primer binding sites to the HIV-1 target RNA, but are distinguished from HIV-1 target amplicon through a unique probe binding region. The QS is a non-infectious Armored RNA construct that was added to the sample at a known copy number. Protease, lysis reagent and magnetic glass particles were added to the sample to lyse the HIV-1 virus particles, release nucleic acids and protect the released HIV-1 RNA from RNases in plasma. The HIV-1 RNA and QS RNA bound to the surface of the glass particles and unbound salts and proteins were removed by washing. After separation and washing, the adsorbed HIV-1 RNA and QS RNA were eluted with an aqueous solution. The sample was added to an amplification mixture and transferred to the COBAS® TaqMan® Analyser. HIV-1 RNA and QS RNA were reverse transcribed, amplified using PCR and detected using dual-labelled fluorescent probes. The COBAS® TaqMan® Analyser compared the HIV-1 signal to the HIV-1 QS signal for each sample and control and calculated the HIV-1 RNA concentration (Roche Diagnostics, 2013).

Appendix 11 Determination of plasma adiponectin

Sample requirements

The sample was taken into an EDTA tube. The Quantikine Adiponectin ELISA kit (R & D Systems) used 50 µl of sample for the test.

Methods

All samples were pre-diluted 1/100 before performing the assay. 100 µl of proprietary assay diluent was added to each well of a microplate. To this, 50 µl of standard, control, or sample (pre-diluted) were added to the respective wells. The plate was sealed with an adhesive strip. The plate was incubated for 2 hours at room temperature. The contents of each well were aspirated and the wells washed four times with 400 µl proprietary wash buffer. 200 µl of adiponectin conjugate was added to each well. The wells were covered with a new adhesive strip and incubated for 2 hours at room temperature. The aspiration and washing step was repeated. 200 µl of substrate solution was then added to each well and the plate was incubated for 30 minutes at room temperature protected from the light. Stop solution (50 µl) was added to each well inducing a colour change from blue to yellow. The optical density of each well was determined using a microplate reader set to 450 nm. Intra-assay CVs were 2.5% and 4.7% and inter-assay CVs were 6.8% and 6.9%.

Appendix 12 Determination of plasma cytokines

Sample requirements

The sample was taken into an EDTA tube. The Evidence Investigator™ Cytokine & Growth Factors High-Sensitivity Array (Randox, UK) used 250 µl of sample for the test.

Methods

Proprietary assay buffer was pipetted into each well of a biochip. The calibrator, sample, or control was added to their respective wells. The handling tray containing the biochip was incubated for 1 hour at 37°C and 370 rpm. Following this, the wells were covered and placed at 2-8°C for 16-20 hours. Following overnight incubation, the cover strips were removed and the reagents discarded. Two quick wash cycles were carried out using proprietary wash buffer added to each well. This was followed by four further wash cycles and incubation in wash buffer for 2 minutes. After the final wash, residual wash buffer was removed from the biochip carrier by flicking the handling tray. Conjugate was added to each well and the reagents within the well were mixed. The biochip was incubated for 1 hour at 37°C and 370 rpm. Following incubation, the reagents were discarded and washing was carried out as described previously. After the final wash, the wells were filled with wash buffer and left to soak for up to 30 minutes prior to imaging. The biochip to be imaged was removed from the handling tray. The wash buffer was removed and working signal reagent was added to each well. The biochip was placed into the Evidence Investigator™ automated analyser after exactly 2 minutes. Images were captured automatically using dedicated software. The light signal generated from each of the test regions on the biochip was detected using digital imaging technology and compared to that of a stored calibration curve. The concentration of analyte present in the sample was calculated from the calibration curve. The sensitivity of the assay for each analyte (ng/L) is shown below.

IL2	IL4	IL6	IL8	IL10	VEGF	INF γ	TNF α	IL1 α	IL1 β	MCP1	EGF
0.9	2.12	0.12	0.36	0.37	1.53	0.44	0.59	0.19	0.26	0.66	1.04